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June 24/05
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: J. Clark & C. Denning

Filing Date: June 13, 2000

Serial No: 09/593,316

Docket: 730/002

Title: ANIMAL TISSUE FOR XENOTRANSPLANTATION

Art Unit: 1632

Examiner: Quan J. Li, Ph.D.

**APPEAL BRIEF**

Commissioner for Patents and Trademarks
Alexandria, VA 22313

Dear Sir,

Applicant hereby appeals from the final Office Action Mailed December 16, 2004, rejecting all claims under examination in this application. This paper constitutes applicant's Appeal Brief, as required under 37 CFR § 41.37.

A Notice of Appeal was filed in this application on December 24, 2004, setting the deadline for filing an Appeal Brief to February 24, 2004. This Brief is accompanied by a request for a four-month extension of time, along with authorization to charge the Deposit Account with the requisite fees, setting the due date to June 24, 2004. Accordingly, this paper is timely filed.

The Board of Patent Appeals and Interferences is respectfully requested to reverse rejection of the claims and allow the patent to issue, in view of the following remarks.

## STATEMENTS PURSUANT TO 37 CFR § 1.192

Real Party in Interest

The real party in interest for the claimed invention is Geron Corporation, a Delaware corporation, to which the application and the claimed invention has been assigned in their entirety.

Related Appeals and Interferences

No other appeals or interferences are known by applicant or its representative that would directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

Status of claims

- Claims 1-6: Under examination: Rejected under § 101 and § 112 ¶ 1
- Claim 7: Withdrawn
- Claims 8-12: Cancelled
- Claims 13-16: Under examination: Rejected under § 112 ¶ 1
- Claim 17: Withdrawn
- Claims 18-21: Cancelled
- Claim 22: Withdrawn
- Claims 23-26: Cancelled
- Claims 27-32: Withdrawn
- Claims 33-37: Under examination: Rejected under § 101 and § 112 ¶ 1

Status of amendments

All amendments to the claims have been entered. No amendments were filed after the last final Office Action.

Grounds of rejection to be reviewed on appeal

Claims 1-6, 13-16, and 33-37 stand rejected under the enablement requirement of 35 USC § 112 ¶ 1. The basis of this rejection is essentially that the specification fails to provide a working example of a homozygous α1,3GT knockout adult sheep (even though some of the claims refer only to isolated tissues or cells, such as could be created in culture).

The specification provides the sheep α1,3GT gene sequence for the first time, which enables the reader to make α1,3GT knockout cells, tissues, and animals according to standard methods used in other species. The standard methods needed for the reader to carry out the full scope of the claimed invention are referenced in the specification. Nevertheless, the Examiner has maintained that the specification is not enabling without a working example because the relevant art is too unpredictable.

Claim 16 also stands rejected under the enablement requirement of § 112 ¶ 1 on the assertion that the α1,3GT knockout tissue of the invention does not solve all the problem of xenotransplantation.

Claims 1-6 and 33-37 stand rejected under 35 USC § 101 as having no credible utility. The basis of this rejection is essentially that the claimed invention is not enabled, and so cannot have any utility.

History of the application

The claims under examination were initially rejected for not having a working example under the written description requirement of § 112 ¶ 1. Applicant appealed on June 4, 2003. The application was pulled back into regular prosecution on January 30, 2004, so that the Examiner could make essentially the same rejection under the enablement requirement of § 112 ¶ 1, and the utility requirement of § 101. The written description rejection has been withdrawn.

## ARGUMENT

This application provides the sequence for the sheep α(1,3)galactosyltransferase ("α1,3GT") gene, which was cloned and characterized for the purpose of making α1,3GT sheep. α1,3GT knockout mice had already been produced in other labs, and efforts to make α1,3GT pigs were already under way. Using genetically modified stem cells (the technique used to make the mice) is not amenable to making knockout sheep — but nuclear transfer cloning in sheep was a well established technology at the time of filing, and has been used since by several labs to make α1,3GT knockout pigs.

The sheep sequence is the latchkey that enables knockout sheep to be made in the same fashion as other animals. This is the kernel of the inventive aspects of this disclosure, and places into the hands of the public the entire scope of the claimed invention by using standard technology.

The Examiner has taken the position that none of the claims of this invention can be enabled without a working example of a homozygous α1,3GT knockout adult sheep (even though some of the claims refer only to isolated tissues or cells, such as could be created in culture). In support of this position, the Examiner has selectively interpreted published academic articles to indicate that animal cloning is a difficult and unpredictable field.

Geron Corporation, as applicant for the claimed invention, does not deny that cloning is both time-consuming and expensive. However, neither of these aspects imply that completion of the project would require undue experimentation defined by *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). In fact, Geron decided to stop funding of this project part way through because of the costs involved. Preliminary results of the project (cloned α1,3GT knockout fetuses) were then published in an academic journal (Denning et al., Nat. Biotechnol 19:559, 2001) — not as evidence of failure, but to demonstrate the progress made. Applicant has no doubt that the making of α1,3GT knockout sheep can be completed as described in the application as a mater of routine experimentation when the project is resumed. Naturally, the delay in obtaining a patent for this invention is not helpful in obtaining the financial support of another backer.

It is applicant's position that rejection of the claimed invention under 35 USC §§ 101 and 112 ¶ 1 for lack of working examples of all embodiments of the invention is improper.

An instructive parallel can be drawn from the patentability requirement for claims covering human treatment, and the manufacture of pharmaceutical products for human use. The

courts and the Office have long recognized that applicant does not need to have a working example of the treatment in a human subject in order for the invention to be patentable.

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of patent laws. *Scott [v. Finney]*, 32 USPQ2d 1115. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily induces the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer. *In re Brana,* 34 USPQ2d 1436 (Fed. Cir. 1995).

Similarly, applicant for the invention claimed here should not be denied patent coverage, just because a homozygous knockout sheep was not obtained before the application was filed. Again, were the patent office require a full working example of the entire scope of this type of invention in order to prove utility and enablement, the associated costs would eliminate an important incentive to create products for use in regenerative medicine and tissue transplantation.

Applicant should not be penalized for disclosing their invention before they completed reduction to practice of all the embodiments. To the contrary: an objective of the patent law is to disseminate advances in the art and thereby provide a public benefit as soon as possible. The inventors of the claimed invention fully entered into the spirit of the public policy objective by filing their patent disclosure soon after critical elements of the invention had been made (isolation and characterization of the sheep α1,3GT gene). They knew full well that this places the invention in the hands of the public through the implementation of standard cloning technology.

For these reasons, applicant respectfully submits that the claimed invention meets all the patentability requirements of 35 USC §§ 101 and 112.

The following remarks address in more detail the arguments made during prosecution.

Summary of the Invention

The claimed invention provides ovine cells, tissues, and animals that have been engineered for reduction or elimination of the carbohydrate epitope Galα(1,3)Gal, which ovine cells normally express. This epitope is important because it is a foreign antigen against which humans have naturally occurring antibody. Upon transplant of tissue bearing this epitope into an animal with naturally occurring antibody causes immediate hyperacute rejection. The idea is that elimination of Galα(1,3)Gal from the tissue will help render it more suitable for use in human transplantation therapy.

The epitope is produced by the enzyme α(1,3)galactosyltransferase (EC 2.4.1.124; abbreviated herein as "α1,3GT"), which adds galactose at the α(1,3) position to membrane-anchored Galβ(1,4)GlcNAc acceptor substance. Inactivation of the α(1,3)galactosyltransferase locus at both of the two alleles (a homozygous knockout) eliminates the enzyme from the cell, which in turn prevents the Galα(1,3)Gal epitope from being formed.

The invention is made possible by the discovery and isolation of the sheep α(1,3)galactosyltransferase (α1,3GT) gene and its genomic clone. The sequence of the gene is provided in the application (Example 1), and clones are deposited with the NCIMB in the United Kingdom in support of this application (page 51).

The specification illustrates how the α1,3GT sequences can be used to create targeting vectors (Example 3), which can then be used to inactivate the α1,3GT gene in isolated sheep fibroblasts from different strains (Examples 4 and 5). Heterozygous (single knockout) animals had been created from the targeted cells by the time the application was filed (Example 6), according to standard techniques in the field of animal cloning. Homozygous knockout animals can be made by using a double knockout cell in the cloning process, or by cross-breeding heterozygous knockout animals (pages 37-41). The cells and tissue made during the course of this work are then characterized (pages 41-43) to verify that they have the characteristics of the claimed invention — either at the genetic level (inactivation of the α1,3GT gene) or at the phenotypic level (reduced expression of the Galα(1,3)Gal epitope).

Rejections under 35 USC § 112 ¶ 1

Claims 1-6, 13-16 and 33-37 stand rejected under the enablement requirement of § 112 ¶. The central issue appears to be the fact that the specification does not indicate that a homozygous α1,3GT knockout sheep actually having been made.

Of course, the Board will recognize that there is no legal requirement that an actual working example be provided in the specification in order for a patent disclosure to be enabling.

> It is well established in the law that a specification can adequately describe the manner and process of making an embodiment of an invention, whether or not it has actually been conducted. Use of prophetic examples does not make a patent non-enabling. The burden is on the person challenging the patent to show . . . that the prophetic examples together with other parts of the specification are not enabling. *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.,* 224 USPQ 409 (Fed. Cir. 1984).
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> Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided though broad terminology or illustrative examples. *In re Wright,* 27 USPQ2d 1510 (Fed. Cir. 1993).

It is applicant's position that the claims are fully described and enabled, *inter alia* because the only elements missing from the working examples can be achieved as a matter of routine experimentation by the skilled reader.

Specifically, the description provides the newly discovered nucleotide sequence of the sheep α1,3GT cDNA (SEQ. ID NO:1) and the α1,3GT gene (SEQ. ID NOs:14 to 25). This is supported by a biological deposit (NCIMB Accession No. 41056) comprising cloned genomic DNA. Example 3 illustrates how the α1,3GT sequences can be used to create targeting vectors, which can then be used to inactivate the α1,3GT gene in isolated sheep fibroblasts from different strains (Examples 4 and 5). Heterozygous (single knockout) fetal sheep were created from the targeted cells by the time the application was filed (Example 6), according to standard techniques in the field of animal cloning.

Homozygous knockout animals can be made by using a double knockout cell in the cloning process, or by cross-breeding heterozygous knockout animals (pages 37-41). The cells and tissue made during the course of this work are then characterized (pages 41-43) to verify that they have the characteristics of the claimed invention — either at the genetic level (knockout of the α1,3GT gene) or at the phenotypic level (reduced expression of the Galα(1,3)Gal epitope). Furthermore, the homozygous knockout cells of claims 4, 5, and 33-37 can be made without

making a homozygous knockout animal, or doing any animal cloning at all (page 40, line 20 to page 41, line 13).

In requiring applicant to provide a working example of a homozygous α1,3GT knockout sheep, the Examiner has relied on the assertion that the area of making knockout animals is generally unpredictable. However, the Examiner has raised no specific technical issue why the making of a knockout sheep should pose special technical difficulties — difficulties that were not experienced in making α1,3GT knockouts in two other animal species.

Accordingly, the Office has not met its burden of establishing a *prima facie* case for non-patentability under 35 USC § 112 ¶ 1.

Nevertheless, in responding to the Office Actions, applicant has explained how it is possible to make a homozygous α1,3GT knockout sheep without undue experimentation, and why the phenotype is predictable.

***1. Sheep cells having an inactivated α1,3GT allele can readily be produced.***

One embodiment of the invention disclosed in this application are sheep cells in which the α1,3GT gene has been inactivated. This is claimed directly in claim 4 and claims 33-37. As explained in the specification, α1,3GT knockout cells can also serve as nuclear donors for the making of knockout animals by nuclear transfer (animal cloning).

The application newly provides the sheep α1,3GT gene, and describes and illustrates how to make targeting constructs for the purposes of creating α1,3GT knockout cells. Methods for using gene sequences to inactivate the corresponding gene in living cells are described extensively in the art.

- *Gene Knockout Protocols,* by Martin J. Tymms (Editor), Ismail Kola (Editor). 431 pages; Humana Press; 1st edition (January 15, 2001).
- *Laboratory Protocols for Conditional Gene Targeting,* by Raul M. Torres, Ralf Kuhn. Oxford University Press (October 1997).
- *Homologous Recombination and Gene Targeting,* by John Sedivy. MacMillan Pub Co. (November 1991).
- *Gene Targeting: A Practical Approach,* by Alexandra L. Joyner (Editor). Oxford University Press; ISBN: 019963792X; 2nd edition (February 15, 2000).

- *The Gene Knockout Factsbook* (2-Volume Set), by Tak W. Mak (Editor), Josef Penninger (Editor), John Roder (Editor), Janet Rossant (Editor), Mary Saunders. 1140 pages; Academic Press; 1st edition (November 15, 1998).
- *Gene Targeting Protocols (Methods in Molecular Biology, Vol 133),* by Eric B. Kmiec (Editor), Dieter C. Gruenert (Editor). Humana Press (January 15, 2000).

Figures 9-15 in the application provide illustrations of targeting constructs based on the α1,3GT sequence that will inactivate the α1,3GT gene by removing exon sequences. Figure 16 illustrates the successful targeting and deletion of Exon 4, using the p0054 vector[1]. In Example 5, similar targeting was demonstrated using the same constructs on a different sheep strain cell line[2].

Thus, the skilled reader may use the constructs provided in the working examples, or design their own knockout strategy, by applying the sheep α1,3GT sequence using standard procedures in order to make the knockout cells of the invention.

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[1] The Office Action of May 21, 2002 (page 7) indicates concern that the specification provides no direct evidence for successful targeting of Exons 8 and 9. Nevertheless, targeting Exons 8 and 9 should be achievable without undue experimentation. In any event, it is not necessary to target Exons 8 and 9 to practice the claimed invention. It is only necessary to eliminate the translation start or remove enough of the gene to prevent the gene product from being functional. The targeting of Exon 4 (as effected in the working examples), or some other portion of the gene, or various portions in combination will be sufficient for the purpose of inactivating the α1,3GT gene in a sheep cell. The enablement requirement is met if the description enables *any mode* of making and using the claimed invention. *Engel Industries, Inc. v. Lockformer Co.,* 20 USPQ2d 1300 (Fed. Cir. 1991), emphasis added.

[2] The Office Action of November 23, 2001 (page 8) says that the specification is enabled for homozygous inactivation of the α1,3GT gene in the Finn Dorset strain of sheep, but not in other strains. It is recognized in the art of homologous recombination that a mismatch of about 1% (i.e., identity of about 99%) is well tolerated when using targeting constructs to cause gene inactivation. The difference between strains of the same mammalian species typically falls within this range. The α1,3GT sequence and targeting constructs used in the working examples were obtained from Black Welsh Mountain fibroblasts (Example 1). They have been used to successfully inactivate the α1,3GT gene in both Black Welsh Mountain sheep cells (Example 4), and Finn Dorset sheep cells (Example 5). This confirms that the claimed invention is enabled for different strains within the ovine species.

*2. Sheep cells that are homozygously inactivated at the α1,3GT locus can readily be produced*

Inactivating a single α1,3GT gene in a cell creates a heterozygous knockout. In order to prevent expression of the Galα(1,3)Gal epitope on the cell surface, both α1,3GT alleles must be inactivated (a homozygous knockout). As described in the specification, homozygous α1,3GT knockout cells according to claims 4 and 33-37 can be made using cultured cells, or by harvesting cells from a homozygous knockout animal.

An extensive description of how to make α1,3GT homozygous knockout cells in culture is provided in the specification beginning on page 40, line 20. Several techniques are explained, including sequential targeting of the two alleles by any one of these methods:

- Using a step-wise increase in antibiotic concentration to knock out both alleles (page 40, lines 24-27, citing U.S. Patent 5,589,369)
- Using two different antibiotics to sequentially knockout each allele (page 40, line 27 to page 41, line 4, referring to targeting constructs shown in Figures 9 and 11)
- Knocking out the first allele, and then retargeting and selecting homozygous knockout cells using an antibody that recognizes the Galα(1,3)Gal epitope (page 41, lines 8-13)

The claims have been rejected under 35 USC § 112 ¶ 1 on the assertion that a α1,3GT knockout sheep cannot be made. Recent Office Actions refer back to publications directed towards animal cloning to support the contention that homozygous α1,3GT knockout cells are difficult to make in culture[3]. On this basis, the Examiner indicates that the claims to knockout

[3] The Advisory Action of May 6, 2003, and subsequent Office Actions refer to the article by Phelps et al., Science 299, 411-414, 2003. Heterozygous α1,3GT knockout pig cells were targeted a second time to obtain homozygous knockout cells, for the purpose of making homozygous knockout pigs. As it turns out, the second allele was inactivated not by homologous recombination, but by a fortuitous mutation event. Of course, this doesn't mean that homologous recombination doesn't' work; only that the single positive event ultimately cloned out of the system happened to have been achieved in an unexpected manner. The Advisory Action quotes col. 3 of page 413, which attributes the finding of the cell with the desired phenotype to a "powerful selection method". The method is described in col. 1 of page 412, and involves selecting targeted cells using a toxin that eliminates cells still expressing the Galα(1,3)Gal epitope. *An equivalent method is described in the specification on page 41, lines 9-11,* which teaches using an epitope-specific antibody to cause complement-mediated lysis of cells still expressing the Galα(1,3)Gal epitope. Thus, the patent application provides all the tools needed for the skilled reader to make a α1,3GT knockout cells in a manner that is equivalent to the Phelps method. As explained in Section 4 below, the Phelps article also confirms that this patent application enables the making of a α1,3GT knockout sheep.

cells are not enabled[4]. Again, the Examiner is essentially requiring applicant to provide evidence of actual reduction to practice in order to meet the requirements of § 112 ¶ 1.

U.S. Patent 5,589,369 referred to in the specification describes and claims a system for making homozygous knockout cells[5]. Since this is an issued patent, 35 USC § 282 requires that the patent be presumed valid, and therefore enabled under 35 USC § 112 ¶ 1. The Examiner has not explained why the making of α1,3GT knockout cells would pose special difficulties that prevent the method of U.S. Patent 5,589,369 (or the other alternatives described in the specification) from being implemented as a matter of routine experimentation.

The steps referred to in Sections 1 and 2 are sufficient to enable the full scope of the cells in claims 4 and 33-37.

### *3. Animals having an inactivated α1,3GT allele can readily be made from α1,3GT inactivated donor cells by nuclear transfer*

Some claims in the application involve or are facilitated by the making of a cloned animal from α1,3GT knockout cells. A central aspect of this invention relates to the discovery and characterization of the sheep α1,3GT gene, and its use for inactivating the α1,3GT gene inside cells. The α1,3GT knockout cells explained in Sections 1 and 2 above can be made into knockout animals using standard methods known in the art.

Several methods are available for making genetically modified animals from genetically altered cells. The specification explains extensively on pages 37-41 that animals can be cloned from a suitable donor cell by nuclear transfer. This is proven technology that created Dolly the sheep. The nuclear transfer method has been fully described and enabled in issued U.S. patents 6,147,276 and 6,252,133 (Campbell & Wilmut, Roslin Institute).

There is no reason to believe that genetically altering the donor cell would affect its suitability as a nuclear donor. To the contrary. A number of published experiments confirm that

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[4] This is contrary to the position taken in the Office Action of November 23, 2001. On page 8, the Office Action says that the specification is enabled for homozygous inactivation of the α1,3GT gene in Finn Dorset sheep.

[5] US 5,589,369 (Seidman and Jakobovits, Cell Genesys) is entitled "Cells homozygous for disrupted target loci". Claim 1 covers "A method for making diploid mammalian cells homozygous for disrupted target loci . . . comprising . . . (a) introducing into diploid mammalian cells a construct . . . comprising a selectable marker gene . . . (b) growing the cells . . . in said selective medium at a first level of selective agent; (c) subjecting the population of cells . . . to a level of selective agent greater that said first level . . . and (d) isolating said cells . . ."

cloned animals may readily be made from genetically altered cells according to the Campbell & Wilmut method.

1. Uchida et al. (Transgenic Research 10:577, 2001) report the production of transgenic miniature pigs by pronuclear microinjection. The Huntington gene cloned from miniature pig, was linked to rat enolase promoter, and injected into pronucleus of fertilized eggs. Several of the offspring were determined to have the transgene by PCR and Southern analysis.
2. Bondoli et al. (Molec. Repro. Dev. 60:189, 2001) report cloned pigs generated from cultured skin fibroblasts derived from a boar with an H-transferase transgene. Two healthy piglets resulted from nuclear transfer by fusion of fibroblasts that had been extensively cultured with enucleated oocytes.
3. Lai et al. (Molec. Repro. Dev. 62:300, 2002) report a transgenic pig expressing green fluorescence protein. Fetal-derived fibroblast cells were transduced with the GFP gene, and then cloned into porcine oocytes. A healthy transgenic piglet was obtained that expressed GFP.
4. McCreath et al. (Nature 405:1004, 2000) report transgenic sheep made by nuclear transfer from fibroblast donors in which different transgenes were targeted into the alpha1(I) procollagen locus.
5. Lai et al. (Science 295:1089, 2002) report production of α(1,3)galactosyltransferase knockout pigs by nuclear transfer cloning. The pigs were produced by nuclear transfer, using clonal fetal fibroblast cell lines as nuclear donors.
6. Dai et al. (Nature Biotech 20:251, 2002) also report production of α(1,3)galactosyltransferase knockout pigs by nuclear transfer cloning. The pig α1,3GT gene was disrupted in both male and female porcine primary fetal fibroblasts, which were then used for nuclear transfer. Six clonal fetal piglets were obtained, of which five were normal weight and apparently healthy. Southern blot analysis confirmed that the five piglets contained one disrupted α1,3GT allele.
7. Denning et al. (Nat. Biotechnol 19:559, 2001) describe the deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Eight pregnancies were maintained to term and four PrP-/+ lambs were born.
8. Schnieke et al. (Science 278:2130, 1997) report production of human factor IX transgenic sheep. Ovine fibroblasts were transfected with the human factor IX gene,

and used as donors for nuclear transfer to enucleated oocytes. Six live transgenic lambs were born, of which three contained the factor IX gene.

9. Cibelli et al. (Science 280:1256, 1998) transfected bovine fibroblasts with a marker gene, which were then fused to enucleated mature oocytes. Out of 28 embryos transferred, three health transgenic calves were isolated.
10. Kuroiwa et al. (Nature Genetics 36:775, 2004) have produced cattle that are homozygous for inactivation of the bovine gene encoding IgM μ-chain (IGHM). Cells were targeted on one allele and used as donors to make heterozygous fetuses. Tissue was harvested, retargeted using *non-isogenic* vectors, and used to make homozygous knockout animals. *Five rounds* of harvesting fetal tissue, genetic modification, and nuclear transfer, produced tissue with this genotype: homozygous inactivation of IGHM, containing a *Cre* transgene, and homozygous inaction of the PRNP gene (responsible for mad cow disease). Nine pregnancies having the five modifications have survived beyond 60 days. Kirin Pharmaceuticals intends to use these animals for producing human IgM antibody for therapy.
11. Ramsoondar et al. (Biol. Reprod. 69:437, 2003) reported the production of pigs containing both a α1,3GT knockout and an α(1,2)fucosyltransferase transgene. Donor fibroblasts *already contained a genetic modification* — the α1,2FT transgene. They were targeted at the α1,3GT locus with *non-isogenic* DNA, producing cells that had two genetic modifications — which were then used successfully for nuclear transfer.
12. Sendai et al. (Transplantation 76:900, 2003) reported production of heterozygous α1,3GT cattle. One fetus was produced from 24 cloned embryos. A fibroblast cell line was established from the fetus for second round targeting, intended for cloning into a homozygous knockout.

These references confirm that pigs, sheep, and cattle can all be cloned by the Campbell & Wilmut method using genetically altered cells to make genetically modified animals. References 5, 6, 7, 11, and 12 are of particular interest, because they illustrate that heterozygous α1,3GT knockout animals can readily be made by nuclear transfer of heterozygous knockout cells. All the evidence of record indicates that the cloning of sheep according to the Campbell & Wilmut method is no more difficult if the cell used as the nuclear donor has been genetically altered.

***4. Animals that are homozygous for inactivated α1,3GT can readily be produced***

The skilled reader has at least three options by which to make a sheep in which both α1,3GT alleles have been inactivated:

1. Homozygous knockout cells can be made in culture as explained in Section 2 above. They are then used as nuclear donors to make homozygous knockout animals by the animal cloning method of Campbell & Wilmut (specification: page 37-41).
2. As an alternative, a nuclear donor cell with α1,3GT inactivated on one allele is used to produce a heterozygous knockout animal. Cells are harvested for a second round of targeting. This generates homozygous knockout cells, which can then be used to generate homozygous knockout animals by a second cloning event (specification: page 41, line 17). The article by Kuroiwa et al., *supra,* provides an illustration of genetically modified cattle made by five rounds of sequential cloning.
3. Another alternative again involves making a heterozygous knockout animal to start. However, in this case, heterozygous knockout animals are simply cross-bred to produce a homozygous knockout animal (specification: page 41, line 14). This requires time for breeding the second generation, but in some ways is the most straight-forward option.

Since these technologies are all in wide-spread general use, the only relevant question in relation to the invention claimed in this application is *whether knocking out both α1,3GT alleles would somehow compromise the viability of the animal.*

In fact, we know this not to be the case. Humans and other Catarrhine primates are exceptions amongst mammalian species as not having an expressed α1,3GT gene. We seem to get along quite well without it. The α1,3GT gene has been obtained from two other species that normally express it, and used to create homozygous knockouts without difficulty.

Furthermore, the α1,3GT gene has successfully been knocked out in at least two other mammalian species that normally express it.

- U.S. Patent 5,849,991 (Cols. 48-57) describes the isolation of the mouse α1,3GT gene, and then using it to make homozygous α1,3GT knockout mice.
- Phelps et al., Science 299, 411-414, 2003 describe production of homozygous α1,3GT knockout pigs using the pig α1,3GT gene[6].
- Kolber-Simonds et al. at Immerge BioTherapeutics (Proc. Natl. Acad. Sci. USA 101:7335, 2004) are *another group* to report production of *homozygous α1,3GT knockout pigs.*

The α1,3GT knockout mice of the '991 patent were made by targeting one α1,3GT locus in mouse embryos to make heterozygous knockout, and then cross-breeding to obtaining the homozygous knockouts (option 3, above). Such mice have been used extensively in labs around the world for immunological and transplant studies, and have the usual features of animals of the murine species — with the exception that they lack the Galα(1,3)Gal epitope on their cells.

The knockout pigs of Phelps et al. were also made according to the methods described in this patent application (option 2). First, the pig α1,3GT gene was used to make heterozygous knockout donor cells, which were then used to clone heterozygous knockout pig (page 412, col. 1; described in the present application *inter alia* on page 38, line 5 to page 40, line 19; and page 41, line 22 to page 42, line 5). Next, homozygous knockout cells were made by a targeting the other allele in the donor cells using a knockout vector, and selecting cells deficient in the Galα(1,3)Gal surface antigen (page 412, col. 1; described in the present application *inter alia* on page 41, lines 9-13 and 17-20; and page 42, lines 6-16). Finally, double knockout cells were used as donor cells for nuclear transfer to produce homozygous knockout animals (abstract; described in the present application *inter alia* on page 38, line 9 to page 40, line 19).

Four double-targeted female piglets were produced by Phelps et al., of which three had α1,3GT inactivated on both alleles (page 412, col. 3 ff).

Similarly, the knockout pigs of Kolber-Simonds et al. were made by knocking out the two α1,3GT alleles in two sequential rounds of cloning. Cell lines established from heterozygous

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[6] The pig α1,3GT gene sequence had already been disclosed in U.S. Patent 5,821,117. This patent includes claims both to the gene sequence *and to cells having an inactivated α1,3GT gene.*

knockout cells were selected for spontaneous inactivation of the second allele using antibody staining. They were then used successfully as nuclear donors: 48 transfers resulted in 17 pregnancies, and 4 homozygous α1,3GT knockout piglets.

Based on the precedents of humans, other Catarrhine primates, and homozygous knockout mice, and pigs, there is no reason to believe that homozygous knockout sheep would not be equally viable, and equally straight-forward to produce by any one of the three approaches described in the specification.

Nevertheless, in the Office Action dated January 30, 2004 states that *contrary to the pig, knockout of α1,3GT gene kills ovine fetuses* (page 17). This statement is entirely without foundation. The Galα(1,3)Gal antigen has no known biological function that is required for survival. All upper primates lack α1,3GT, and seem to get along quite well without it. Homozygous α1,3GT knockouts have been made in the mouse and the pig without difficulty. There is no evidence of record to indicate that sheep are any different.

The notion that knocking out the α1,3GT gene will specifically kill sheep is not substantiated in any cited reference, and appears to come from the personal knowledge or speculation of the Examiner. For this reason, applicant made a formal request for an Examiner's Affidavit on August 2, 2004, pursuant to 37 CFR § 1.104(d)(2) and MPEP § 2144.03. This request was denied, but the same assertion was made in the Office Action dated December 24, 2004.

***5. Cells from homozygous knockout animals will have cells and tissues lacking the Galα(1,3)Gal xenoantigen***

As described in the specification, the α1,3GT gene is responsible for forming the Galα(1,3)Gal xenoantigen in non-Catarrhine mammals. An animal that is homozygous for inactivation of the α1,3GT gene would therefore lack the enzyme responsible for making the Galα(1,3)Gal epitope.

The homozygous knockout mice in U.S. Patent 5,849,991 confirm this expectation. Peripheral blood monocytes and splenocytes from the homozygous knockouts were analyzed for presence of the Galα(1,3)Gal antigen using the IB4 lectin, in a manner comparable to what is described in the specification of the present application on pages 41-43. Wild-type mice showed high degree of staining, while knockout mice showed minimal staining, confirming that the tissue

was essentially devoid of the Galα(1,3)Gal antigen (U.S. 5,849,991, Cols. 48-52). As expected, since Galα(1,3)Gal is not a self-antigen in these mice, they form naturally occurring antibody against it, as do humans (Chong et al., Transpl Immunol 8:129-37, 2000).

The homozygous knockout pigs in the article by Phelps et al. This is shown in are devoid of antibody-detectable Galα(1,3)Gal. See Fig. 1., clones B1-1, B1-2, and B1-4 (the three correctly targeted clones), and Fig. 2.

Similarly, Dor et al. (Transplantation 78:15, 2004) showed that the five α1,3GT knockout pigs made in the manner of Kolber-Simonds et al. had essentially no expression of Galα(1,3)Gal as determined by IB4 staining (Table 1, Figure 1) and have naturally occurring cytotoxic anti-Galα(1,3)Gal antibody (Figure 3).

Apparently the Examiner agrees that homozygous α1,3GT knockout sheep will lack the Galα(1,3)Gal antigen on their cells. The Office Action dated December 24, 2004 (page 17) states: "This is not an issue once a homozygous α1,3GT knockout animal is made."

***Predictability of the Art***

In requiring applicant to provide full reduction to practice of a homozygous α1,3GT knockout sheep, the Examiner asserts that the "physiological art" in general, and the art of making genetically altered animals in particular, is unpredictable. In support of this assertion, two publications have been put forward in the Office Actions.

The Office Action of November 23, 2001, refers to an article by Linder et al. (Lab. Anim. NY 30:34, 2001) as indicating that the resulting phenotype of a targeted gene mutation would vary among different strains of animals because of the collective effect of different genes in the host. The issue raised in the article is that inactivation of a gene may generate different phenotypes in particular inbred strains of mice. This is because the genes exemplified do not directly generate the phenotype being measured, but cause the phenotype to change by complex interaction with other gene products.

In contrast, the α1,3GT gene targeted in the present invention is directly responsible for generating the enzyme that builds the Galα(1,3)Gal epitope[7]. Accordingly, none of the concerns raised in the Linger article are relevant. The homozygous α1,3GT knockout mice described in U.S. Patent 5,849,991 were made by breading heterozygous knockout mice, and the line has continued to bred true. All evidence indicates that α1,3GT knock-out animals reliably breed towards absence of the Galα(1,3)Gal epitope, as expected.

The Advisory Action of May 6, 2003, refers to the article by Denning, Clark, et al. (coinventors on this patent application) entitled *Deletion of the α(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in the sheep* (Nature Biotechnol. 19:559, 2001). The Examiner is apparently concerned that the first α1,3GT knockout sheep clones died *in utero*. But the Denning article was only a preliminary report. It does not support the contention that gene targeting in sheep is an uncertain process.

On the contrary — the article provides several illustrations of the viability of the claimed invention:

- Sheep cells can be correctly targeted for inactivation of the α1,3GT gene. See Figure 2, panel (A); and Table 1. This provides a direct illustration of the making of the knockout cells covered by claims 4 and 33-37.
- Targeted cells can be used for nuclear transfer. There are three examples: a) the α1,3GT knockout cell gave rise to viable embryos; b) the PRP knockout cells gave rise to 3 live births; c) viable animals have been produced that were successfully targeted at the COL1A1 locus (ref. 5, discussed on page 559, col. 1).

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[7] Table 2 in the Linder article shows that knocking out the gene for IL-2 can cause splenomegaly, inflammatory bowel disease, or generalized autoimmune disease, depending on the genetic background. A similar observation is made for *ob/ob* obese mice, which have a homozygous mutation in the leptin gene. The target genes in these studies are both endocrine molecules (IL-2 and leptin) which mediate a complex response pathway between different cells. In contrast, the present invention is directed at inactivating a gene that puts a terminal sugar residue onto the carbohydrate substrate N-acetyl lactosamine, which all ovine animals express. Accordingly, no inter-strain variation is expected. Another issue raised in the Linder article is that a cross-over event that occurs during breeding may separate a mutant gene from the phenotype being used to follow the breeding, if the phenotype is not directly encoded by the mutated gene. This is not a concern for the present invention, because the presence of an inactivated target gene can be detected directly — either by PCR analysis (Figures 16 and 17), or by detection of the Galα(1,3)Gal epitope on the animal's cells.

- *Knocking out the α1,3GT gene does not decrease viability of the embryo.* See the data in Table 2. Nuclear transfer with untransfected donor cells (7G65F4) gave rise to 5 viable fetuses at day 60 in 33 attempts (a 13% success rate). Nuclear transfer of cells treated with the α1,3GT vector but not inactivated (4H2) gave rise to 2 viable fetuses in 23 attempts (an 8% success rate). Nuclear transfer of cells containing an inactivated α1,3GT gene (3C6 and 5E1) gave rise to 5 viable fetuses in 21 attempts (a 19% success rate). Ergo, the success rate for cloning sheep by nuclear transfer is not further reduced by knocking out the α1,3GT gene. If anything, there was actually an improvement in cloning frequency using the correctly targeted cells.

Thus, the Denning article confirms that the generation of α1,3G knockout sheep *poses no undue difficulty* beyond what is usually entailed in producing cloned knockout mammals by nuclear transfer. The method has been used successfully for recombination at the COL1A1 locus in sheep (McCreath et al., Nature 405:1066, 2000), and the α1,3GT locus in pigs (Phelps et al., *supra*).

The sheep α1,3GT knockout project reported in the Denning article lost its funding, and it is for this reason that knockout sheep were not ultimately produced by these authors. There is no question that large animal cloning is a costly and time-consuming process, whether or not *the nuclear donor cell has any kind of genetic alteration.* But that does not mean that the claimed invention is in any way inadequately described or enabled. The cloning step required to complete the invention claimed in this application, while costly, is entirely straight forward. *It can be accomplished without undue experimentation,* well within the Wands standard[8]. There is nothing missing from the specification that the skilled reader needs in order to put this invention into practice[9].

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[8] *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). In *Wands,* the patent application claimed monoclonal antibodies of a particular specificity and affinity. The PTO contended that only 2.8% of the hybridomas obtained were proven to fall within the claim, and thus the claim was not enabled. The Court held that Wands was fully enabled, because it was standard practice to screen negative hybridomas in order to find one that makes the desired antibody.

[9] Except, of course, funding. But 35 USC § 112 ¶ 1 only requires the applicant to provide the skilled reader with the knowledge required to make and use the invention — not the financial resources that may be needed to complete the project.

*§ 1.132 Declaration by Dr. Ian Wilmut*

A Declaration by Ian Wilmut, Ph.D., O.B.E., F.R.S., was filed in this application under 37 CFR § 1.132 on September 23, 2004. A copy of the Declaration accompanies this Appeal Brief. It provides further support for applicant's position that the claimed invention is enabled by the application as filed.

Dr. Wilmut explains that genetically modified animals can readily be made by the methods described in U.S. patents describing nuclear transfer, and provides a number of illustrations. He also explains that the early death of α(1,3)galactosyltransferase knockout sheep fetuses reported in the Denning article is often observed in cloned animals, and not attributable to an effect from the α1,3GT gene. He explains that the sheep α1,3GT gene provided in this application can be used to make α1,3GT knockout sheep, just as the pig α1,3GT gene has been used to make α1,3GT knockout pigs.

Thus, even if it takes several attempts to obtain a working example because of a low frequency of successful cloning events, there is no undue experimentation involved. The skilled reader simply repeats the procedure until a α1,3GT knockout sheep is obtained.

***Patentability of individual claims***

As already explained, techniques suitable for preparing α1,3GT knockout animals are generally known in the art, and referenced in the specification. It has not previously been possible to make α1,3GT knockout sheep, simply because the sheep α1,3GT gene was not previously available. Now that the sheep α1,3GT gene has been discovered and characterized, it is straight forward to produce sheep tissue which is devoid of Galα(1,3)Gal, or which has been inactivated for the α1,3GT gene on one or both alleles, using techniques already proven to be effective in the mouse and the pig.

Claims 6, 14, and 15 cover an ovine animal that is homozygous for inactivation of the α1,3GT gene. They meet the enablement requirements of 35 USC § 112 ¶ 1 for reasons already explained. Briefly, the specification provides the sheep α1,3GT gene, α1,3GT targeting constructs, and α1,3GT knockout sheep cells. α1,3GT knockout sheep can be made by applying standard animal production technology referred to in the specification.

Claims 4 has different requirements under 35 USC § 112 ¶ 1. Heterozygous and homozygous cells can be made in culture without producing a knockout animal.

Claims 33-37 specify that the cell of claim 4 is a fibroblast, kidney cell, hepatocyte (liver cell), cardiac cell, or islet cell. Fibroblasts having the required property, made by homologous recombination of cultured cells, are shown in the specification as working Examples 4 and 5 (page 46 ff.). The other cell types can be produced by homologous recombination using the α1,3GT targeting construct with cultured cells of the particular tissue type, in the same fashion.

Claim 5 depends from claim 4, and requires only that the process for making the cell involve at least one nuclear transfer event. This may involve the making of a cloned animal, or just the making of a single cell or cell culture by nuclear transfer.

Claim 13 depends from claim 4, and covers the use of heterozygous or homozygous α1,3GT knockout cells for making homozygous α1,3GT knockout sheep.

Claim 3 covers a cell or tissue that does not express α1,3GT. Claims 1 and 2 cover tissue devoid of the Galα(1,3)Gal epitope. Again, cells having these properties can be harvested from a α1,3GT knockout animal, or produced *in vitro* by genetic manipulation of cultured cells.

Claim 16 depends from claim 1, calling out a particular use of the cells of claim 1 for transplanting into a mammal having circulating antibody against Galα(1,3)Gal determinants — e.g., a non-cattharine primate, or a α1,3GT knockout mouse or pig.

***Summary***

The Office has not established a prima facie case for lack of enablement for the claimed invention. Heterozygous knockout cells and sheep have been made using the α1,3GT targeting vectors described in the specification. There is no basis to believe that making a homozygous knockout will compromise the viability of the animal in any way. In fact, there is abundant evidence from other species that the making α1,3GT knockouts is straight forward.

Applicant should not be required to provide complete actual reduction to practice in order to demonstrate enablement of the invention, since this is not the legal standard. Methods needed to practice the full scope of the claimed invention are known in the art, and can be implemented by the skilled reader without undue experimentation. The claimed invention is placed into the hands of the public because the critical component needed to make this work in sheep — namely the sheep α1,3GT gene — is provided in the disclosure for the first time.

Thus, the claimed invention is fully described and enabled in the specification, thereby complying with the patentability requirements of 35 USC § 112 ¶ 1.

Further rejection of Claim 16 under § 112 ¶ 1

Claim 16 also stands rejected under 35 USC § 112 ¶ 1 as not being enabled by the specification, because even if ovine tissue devoid of Galα(1,3)Gal determinants could be produced, it would not solve all of the issues that trigger a xenograft response.

Applicant disagrees. The application solves the problem of the Galα(1,3)Gal present on sheep tissue, which would generate hyperacute rejection upon transplantation to a human. The skilled reader would recognize that other issues in transplantation therapy should also be addressed, for example, by the use of immunosuppression and other supportive care that are standard in the transplantation setting. Standard immunosuppressive drugs such as cyclosporin A have a long established track record for overcoming immune rejection of grafted organs (Braun, J. Clin. Apheresis 18:141, 2003; Mueller, Ann. Thorac. Surg. 77:354, 2004), and would be adopted as a matter of course in the use of the claimed invention.

In fact, xenografting has been an established protocol for cardiac valve replacement for almost 40 years: O'Brien et al, Lancet 1:929, 1967. Long-term postoperative survival rates have been between 78% and 94%, depending on what procedure is performed (Stinson et al., J. Thorac. Cardiovasc. Surg. 73:54, 1977; Angell et al., Ann. Thorac. Surg. 28:537, 1979). The success is not impaired by the fact that the value tissue used is taken from animals that normally express Galα(1,3)Gal. The Office Action does not explain why the tissue of this invention would not be effective for therapeutic applications such as cardiac valve therapy — regardless of the epitopes expressed.

Furthermore, Costa et al. (FASEB J. 17:109, 2003) reported experiments in which xenograft survival was tested in a model where cartilage was transplanted from transgenic pigs to α1,3GT knockout mice. The pig tissue expressed the transgene α(1,2)fucosyltransferase which reduces but does not eliminate expression of Galα(1,3)Gal. Control pig cartilage grafted into the mice was rejected in several weeks in a cell mediated response. In contrast, the mice receiving the tissue with the transgene showed a markedly reduced anti-pig antibody response and no Galα(1,3)Gal elicited antibody response. There was a mild cellular infiltrate that was confined to the graft periphery, conferring resistance to delayed rejection. In a cardiac transplant model, Chen

et al. (C.G. Chen et al. Transplantation 65:832, 1998) showed that α1,2FT transgenic and α1,3GT knockout tissue were both protected against hyperacute rejection.

It can therefore be anticipated that the use of α1,3GT knockout tissue (having an even lower level of Galα(1,3)Gal antigen), in combination with standard immunosuppressive regimens, will enable engraftment and survival of therapeutically important grafts according to the claimed invention.

Rejection under 35 USC § 101

Claims 1-6 and 33-37 stand rejected under § 101 as claiming subject matter without a credible asserted or a well-established utility.

In making a rejection under this Section, the Examiner referred to the Office's Utility Guidelines[10] as tying the utility requirement of § 101 to the enablement requirement of § 112 ¶ 1. In fact, the standard referred to is that inventions lacking utility are not considered enabled — not *vice versa.* All the arguments made under this heading in the Office Actions are essentially enablement rejections — again, because of the lack of a full working example in the specification.

The courts have repeatedly found that the mere identification of a pharmacological activity relevant to an asserted use provides an immediate benefit to the public, thereby satisfying the utility requirement.

> Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. . . . Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many compounds as possible, we conclude that adequate proof of any such activity constitutes a showing of practical utility. *Nelson v. Bowler*, 206 USPQ 881, 883 (CCPA 1980).

This is true even if the pharmaceutical agent is in a very early stage of development. *Cross v. Iizuka,* 224 USPQ 739 (Fed. Cir. 1985).

As indicated throughout the specification, ovine tissue devoid of antibody-detectable Galα(1,3)Gal determinants are useful and under development in a number of laboratories for use in xenotransplantation.

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[10] Examination Guidelines for Utility Requirement, B2(2), Federal Register Vol 66(4), published January 5, 2001. Referred to in the Office Action dated December 16, 2004, page 4.

The Office Action indicates that the claimed invention has no utility with respect to heterozygous α1,3GT knockout animals and tissues, because there is no phenotypic difference from normal ovine animals and tissues. However, the specification teaches on page 41, lines 14-17 that heterozygous α1,3GT knockout animals have utility for making homozygous knockout animals by cross-breeding. The use of heterozygous knockout animals to breed a homozygous knockout animal is covered in Claim 13, which has not been rejected under § 101. Crossbreeding of heterozygous knockouts has been used successfully to generate α1,3GT knockouts in mice (U.S. Patent No. 5,849,991).

Furthermore, the specification teaches on page 41, lines 17-20 that tissue from heterozygous α1,3GT knockout animals (both birthed animals and fetuses) have utility for targeting the second allele, thereby obtaining homozygous α1,3GT knockout cells. This in turn can be used for nuclear transfer for production of homozygous knockout animals. Second allele targeting and recloning has been used successfully to generate α1,3GT knockouts in pigs (Phelps et al., Science 299, 411-414, 2003; Kolber-Simonds et al., Proc. Natl. Acad. Sci. USA 101:7335, 2004).

Thus, there are several asserted and credible utilities of α1,3GT heterozygous cells and animals that meet the requirements of § 101.

Applicant respectfully requests that rejection of all claims under examination be reversed, and that the application be allowed and sent to the issue branch without further delay.

Should the Patent Office determine that an extension of time or any other relief is required for further consideration of this application, applicant hereby petitions for such relief, and authorizes the Commissioner to charge the cost of such petitions and other fees due in connection with the filing of these papers to Deposit Account No. 07-1139, referencing the docket number indicated above.

Respectfully submitted,

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June 24, 2005

## CLAIMS APPENDIX

1. Ovine tissue devoid of antibody-detectable Gal$\alpha$(1,3)Gal determinants.

2. The tissue of claim 1, which is selected from the group consisting of lung tissue, kidney tissue, liver tissue, cardiac tissue, pancreatic tissue, and ocular tissue.

3. Isolated ovine cell or tissue that expresses glycosyl transferase enzymes but does not detectably express $\alpha$(1,3)galactosyltransferase ($\alpha$1,3GT).

4. An ovine cell which is heterozygous or homozygous for inactivation of an $\alpha$1,3GT gene.

5. The cell of claim 4, produced by transfer of a nucleus from a donor cell heterozygous or homozygous for inactivation of an $\alpha$1,3GT gene, to an enucleated recipient cell.

6. An ovine animal that is homozygous for inactivation of an $\alpha$1,3GT gene.

7. *(Withdrawn)* A polynucleotide construct effective for inactivating an $\alpha$1,3GT gene in an ovine cell.

8 to 12. *Cancelled*

13. A method for producing an ovine that is homozygous for inactivation of an $\alpha$1,3GT gene, comprising providing an ovine embryo of cells according to claim 4, engrafting the embryo into the uterus of a female, birthing an ovine with an inactivated $\alpha$1,3GT gene from the engrafted female, and if the birthed ovine has the $\alpha$1,3GT gene inactivated on only one allele, then mating it with another ovine with an inactivated $\alpha$1,3GT gene, thereby producing an ovine that is homozygous for inactivation of the $\alpha$1,3GT gene.

14. A method for producing an isolated ovine cell that expresses glycosyl transferase enzymes but does not detectably express $\alpha$1,3GT, comprising isolating the cell from an ovine homozygous for inactivation of an $\alpha$1,3GT gene according to claim 6.

15. A method for producing ovine tissue devoid of antibody-detectable Gal$\alpha$(1,3)Gal determinants, comprising harvesting the tissue from an ovine homozygous for inactivation of an $\alpha$1,3GT gene according to claim 6.

16. A method of xenotransplantation, comprising transplanting tissue devoid of antibody-detectable Gal$\alpha$(1,3)Gal determinants according to claim 1 into a mammal having circulating antibody against Gal$\alpha$(1,3)Gal determinants.

17. *(Withdrawn)* An isolated polynucleotide that comprises a sequence of at least 30 consecutive nucleotides with at least one of the following properties:

a) it is contained in SEQ. ID NO:1 or any of SEQ. ID NOs:14 to 25, but not in any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13;

b) it is contained in phage **B**, **C** and **G** deposited under Accession Nos. NCIMB 41056, 41059, 41060, and 41061; but not in $\lambda$-phage or any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13; or

c) it hybridizes under stringent conditions to a polynucleotide with the sequence in SEQ. ID NO:1 or any of SEQ. ID NOs:14 to 25, but not to a polynucleotide with the sequence in any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13

18 to 21. *Cancelled*

22. *(Withdrawn)* An isolated polypeptide that comprises a sequence of at least 10 consecutive amino acids with at least one of the following properties:

a) it is contained in SEQ. ID NO:2 but not in any of SEQ. ID NOs: 4, 6, 8, 10, and 12;

b) it is encoded in phage **B**, **C** and **G** deposited under Accession Nos. NCIMB 41056, 41059, 41060, and 41061., but not encoded in $\lambda$-phage or present in any of SEQ. ID NOs: 4, 6, 8, 10, and 12; or

c) it is at least 80% identical to 15 consecutive amino acids contained in SEQ. ID NO:2, wherein said sequence is not present in any of SEQ. ID NOs: 4, 6, 8, 10, and 12

23 to 26. *Cancelled*

27. *(Withdrawn)* An isolated polynucleotide comprising a sequence encoding a polypeptide according to claim 22.

28. *(Withdrawn)* An isolated polyclonal antibody or a monoclonal antibody that binds specifically to a polypeptide with the sequence SEQ. ID NO:2 but not to a peptide with the sequence present in any of SEQ. ID NOs: 4, 6, 8, or 10.

29. *(Withdrawn)* An assay method for determining α1,3GT expression by a cell, comprising contacting a polynucleotide according to claim 17 with the cell or with mRNA or cDNA obtained from the cell, detecting any hybrids that form as a result, and correlating presence of the hybrids with expression of α1,3GT by the cell.

30. *(Withdrawn)* A method for producing the antibody specific for sheep α1,3GT, comprising immunizing an animal or contacting an immunocompetent particle with a polypeptide according to claim 22.

31. *(Withdrawn)* A method for preparing a Galα(1,3)Gal determinant, comprising contacting a galactose acceptor saccharide with the polypeptide of claim 26 in the presence of UDP-galactose.

32. *(Withdrawn)* An assay method for determining α1,3GT in a sample, comprising preparing a reaction mixture comprising the sample and an antibody according to claim 28 under conditions that permit the antibody to complex with α1,3GT, and correlating any complex formed with the presence or amount of α1,3GT in the sample.

33. The cell of claim 4, which is a fibroblast.

34. The cell of claim 4, which is a kidney cell.

35. The cell of claim 4, which is a hepatocyte.

36. The cell of claim 4, which is a cardiac cell.

37. The cell of claim 4, which is an islet cell.

## EVIDENCE APPENDIX

- 35 USC § 1.132 Declaration signed by Ian Wilmut, Ph.D., O.B.E., F.R.S., filed in the application on September 23, 2004

- U.S. Patent 5,849,991. *Mice homozygous for an inactivated .alpha.1,3-galactosyl transferase gene.* (Specification, page 3, line 10)

- U.S. Patent 5,821,117. *Xenotransplantation therapies.* (Specification, page 3, line 9)

- Denning et al., Nature Biotechnology 19:559, 2001. *Deletion of the alpha(1,3) galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep.* (IDS of October 22, 2001)

- Phelps et al., Science 299, 411-414, 2003. *Production of α1,3-galactosyltransferase deficient pigs.* (IDS filed March 31, 2003)

- Kolber-Simonds et al., Proc. Natl. Acad. Sci. USA 101:7335, 2004). *Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations.* (IDS filed August 17, 2004)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| Inventors: J. Clark & C. Denning | Art Unit: 1632 |
| Filing Date: June 13, 2000 | Examiner: Qian J. Li, Ph.D. |
| Serial No: 09/593,316 | |
| Docket: 730/002 | |
| Title: ANIMAL TISSUE FOR XENOTRANSPLANTATION | |

**DECLARATION UNDER 37 CFR § 1.132**
**BY IAN WILMUT, Ph.D., O.B.E., F.R.S.**

Commissioner for Patents
Alexandria VA 22313

Dear Sir:

I, IAN WILMUT, do hereby declare as follows:

I am head of the Department of Gene Function and Development at the Roslin Institute in Midlothian, Scotland. The group headed by Keith Campbell and myself cloned Dolly the Sheep — the first mammal to be cloned from an adult cell. The methods we used are described in U.S. Patent Nos. 6,147,276; 6,252,133; and 6,525,243.

I have reviewed the Patent Application by John Clark and Chris Denning referred to at the top of this Declaration. I understand the Examiner has questioned whether genetically qualified animals can be made according to the Campbell and Wilmut method, and whether a homozygous α(1,3)galactosyltransferase knockout sheep can be made using the gene sequence information provided in this patent application.

After the sheep α1,3GT gene was isolated as described in the application, Dr. Clark and Dr. Denning turned their attention to making α1,3GT knockout sheep. Limited resources were available to pursue the project, and the project was not completed. Preliminary results were reported in the research article published by C. Denning et al., Nature Biotech. 19:559, 2004, for which I am a coauthor.

The paper reports that heterozygous α1,3GT knockout cells were produced as donor cells for cloning by nuclear transfer, but no fetus survived the full term of pregnancy. When the longest lived fetus was autopsied, we found abnormalities around the blood vessels in the lung, which were apparently fatal.

Accompanying this Declaration is an article which I coauthored with Susan Rhind et al. (Nature Biotech. 21:744, 2003). The article explains that lung abnormalities of this kind are seen in failed neonatal cloned sheep more often than they are seen in normal sheep pregnancies. Amongst the animals listed in Table 1, animals 1, 3, 4, and 5 were cloned from knockout cells; animal 6 was cloned from a cell containing a randomly integrated transgene, and animals 2, 7, and 8 were cloned from cells without any genetic modification. Lung abnormalities were seen in cases 1, 3, 5, 6, and 7, which means that the abnormality is not attributable to the use of genetically modified cells, but is an artifact of the cloning process in general. We believe the lung abnormalities are due to incomplete reprogramming when the nucleus of the donor cell is transferred to the recipient oocyte during cloning.

The Denning article shows that there was a frequency of failed pregnancies whether they contained a α1,3GT knockout (Table 2, lines 3C6 and 3E1), a randomly integrated gene (4H2), a knockout of the PrP gene (YH6), or were cloned from unaltered cells (7G65F4). Failure of the α1,3GT knockouts was not attributable to the genetic modification, which has no known relationship to the viability of smooth muscle cells. Rather, it reflects the rate of failure in this series of experiments, irrespective of what genetic modifications were made. As explained in the article, we attribute the rate of failure to the number of doublings of the cells in tissue culture.

There is no reason why genetically modified animals cannot be made according to the method that Keith Campbell described in our patent disclosures. Using donor cells that have not undergone extensive culturing, or that have a long replicative capacity may improve the frequency of successful cloning. But it is only the frequency that is affected, not the ultimate efficacy. It is my belief that cultured cell lines such as those used by Denning et al. will successfully generate cloned animals after sufficient persistence.

This is illustrated by the successful cloning of heterozygous and homozygous knockouts and transgenic animals by other laboratories, using the Campbell and Wilmut technique. For example, Phelps et al. (Science 299, 411-414, 2003) and Kolber-Simonds et al. (Proc. Natl. Acad. Sci. USA 101:7335, 2004) have both cloned α1,3GT knockout pigs. Kuroiwa et al. (Nature Genetics 36:775, 2004) cloned cattle that contain homozygous knockouts of both the IgM μ-chain gene, and the PrP gene.

The cloning method used by all these groups is the same as described by Keith Campbell and myself in our U.S. Patents. There is no modification to any aspect of our method — selection of the oocyte, transfer of the nucleus, activation of the combined cell, or implantation into the surrogate female — that is needed for the method to work when the donor cell has been genetically modified.

The patent application by Denning and Clark provides the sheep α1,3GT gene, and describes the making of targeting vectors, and knockout cells. Making α1,3GT knockout sheep from the sheep α1,3GT gene should be no more difficult than making α1,3GT knockout pigs from the pig α1,3GT gene.

PATENT
09/593,316
Docket 730/002

I hereby declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Sept 9, 2004
Date

Ian Wilmut, Ph.D.
Midlothian, Scotland

suggest that there is space for up to three extended chains within the chamber.

Second, little difference in the rates of endoproteolytic cleavage of these disordered substrates was detected between latent 20S and active 26S proteasomes (Figs. 1 to 4) in which the status of the gate that controls entry to the central axial channel of the proteasome is closed and open respectively (Fig. 1C) (*24*, *30*). Physiological regulators of the proteasome, such as PA700 (19S cap) and PA28, increase proteasome activity in part by opening this gate, thereby increasing access of substrates to the proteasome's catalytic centers (*24*, *25*). The ability of closed, latent 20S proteasome to catalyze cleavage of these natively disordered, physiological substrates suggests they possess certain features that also promote "gating" of the proteasome (Fig. 4E), features that folded proteins lack. This mechanism suggests a potential role for the free 20S proteasome found in the absence of bound regulatory proteins in many cells (*33*). It is possible that these inherent signals could target substrates directly for 20S proteasomal degradation without the need for polyubiquitin modification.


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# Production of α1,3-Galactosyltransferase–Deficient Pigs


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The enzyme α1,3-galactosyltransferase (α1,3GT or GGTA1) synthesizes α1,3-galactose (α1,3Gal) epitopes (Galα1,3Galβ1,4GlcNAc-R), which are the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of α1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the α1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the α1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the α1,3GT protein. Four healthy α1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the α1,3GT gene hold significant value, as they would allow production of α1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.


The enzyme α1,3-galactosyltransferase (α1,3GT or GGTA1) synthesizes α1,3Gal epitopes (Galα1,3Galβ1,4GlcNAc-R) on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys (*1*). α1,3Gal epitopes are the major xenoantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation (*2*–*4*). Many reports have also indicated that α1,3Gal epitopes are involved in acute vascular rejection (AVR) of xenografts (*4*–*6*). Piglets with α1,3GT heterozygous knockout have been cloned by our group (*7*) and another team (*8*) in the last year. To produce homozygous α1,3GT knockout piglets by natural breeding, assuming both male and female heterozygous knockout pigs are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and cloning strategy, we could save up to 6 months and all cloned piglets would be α1,3GT double knockout (DKO). We have selected and enriched for α1,3GT DKO cells by using a bacterial toxin, toxin A from *Clostridium difficile*, which binds with high affinity to α1,3Gal epitopes and produces a cytotoxic effect on cells that are α1,3Gal-positive (*9*). Toxin A uses α1,3Gal epitopes as a cell


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surface receptor and causes "rounding" and lifting of the α1,3Gal-positive cells from the surface of the growth vessel (*10*, *11*).

Heterozygous α1,3GT knockout fetal fibroblasts, 657A-I11 1-6 cells, were isolated from a day-32 pregnancy as described in (*7*). To avoid using a second antibiotic-resistance gene as a selection marker, we constructed an ATG (start codon)-targeting α1,3GT knockout vector, pPL680 (*12*), which also contains a *neo* gene, to knock out the second allele of the α1,3GT gene. 657A-I11 1-6 cells were transfected by electroporation with pPL680 and selected for the α1,3Gal-negative phenotype with purified *C. difficile* toxin A (*13*). One colony (680B1) was isolated and expanded after toxin A selection. When the 680B1 cells were stained with a fluorescein-labeled α1,3Gal-specific lectin, GS-IB4, about 80% of the cells were found to be α1,3Gal-negative. The fact that fewer than 100% of the cells in the colony were negative with GS-IB4 staining indicated that this colony contained a mixture of α1,3Gal-negative and -positive cells. We used 680B1 cells for somatic cell nuclear transfer (cloning) as described in (*7*). We transferred embryos to five recipient gilts, and three initial pregnancies were established, of which only one went beyond day 35 of gestation.

To determine whether all the fetuses cloned from 680B1 cells were α1,3GT DKO, we terminated the remaining pregnancy at day 39 and recovered four normal-sized fetuses. Fibroblast cell lines (680B1-1 to B1-4) were isolated from each of these four fetuses, and fluorescence-activated cell sorting (FACS) analysis with GS-IB4 staining showed that B1-1, B1-2, and B1-4 cells were α1,3Gal-negative, whereas B1-3 cells were positive for α1,3Gal (Fig. 1). Normal human serum (NHS) contains preformed antibodies to α1,3Gal and complement proteins, which together cause rapid lysis of cells that are α1,3Gal-positive. A complement lysis assay on these cells showed that B1-1, B1-2, and B1-4 cells were resistant to lysis by NHS, but B1-3 cells were lysed by NHS at the same rate (about 40% of cells lysed) as control wild-type pig cells (Fig. 2). Analysis of genomic DNA from these fetal cells by polymerase chain reaction (PCR) and Southern blot analysis indicated that none of the three α1,3Gal-negative cell lines had the expected restriction fragment pattern predicted for targeted disruption of the second α1,3GT allele with the pPL680 knockout vector. Instead, these fetal cells appeared to have the same allele pattern (one targeted allele and one wild-type allele) as their parent 657A-I11 1-6 cells, which contained only one disrupted α1,3GT allele. Northern blot analysis of the four cell lines (B1-1 to B1-4) showed that they expressed two mRNAs of similar size to those seen in the 657A-I11 1-6 cells (fig. S1). The 3.8-kb band corresponds in size to the wild-type α1,3GT transcript and the shorter 2.5-kb band is the same size expected for the truncated transcript of the first knockout allele (fig. S1). Because of the nature of the toxin A selection method, α1,3Gal-negative cells are selected, regardless of whether inactivation of the second α1,3GT allele was caused by targeted disruption via the pPL680 vector or by any other mechanism. The fact that one normal-sized allele was observed (instead of two shorter knockout alleles) indicated that knockout of the second α1,3GT allele was due to mechanisms other than targeted homologous recombination-mediated disruption, promoter dysfunction, or mRNA missplicing and instability.

To identify the nature of the inactivation event for the second allele, we subcloned and sequenced α1,3GT cDNAs from all four cell lines (B1-1, B1-2, B1-3, and B1-4). Sequencing results revealed that there was a T-to-G transversion at the second base pair of exon 9 in the nontargeted α1,3GT allele of B1-1,

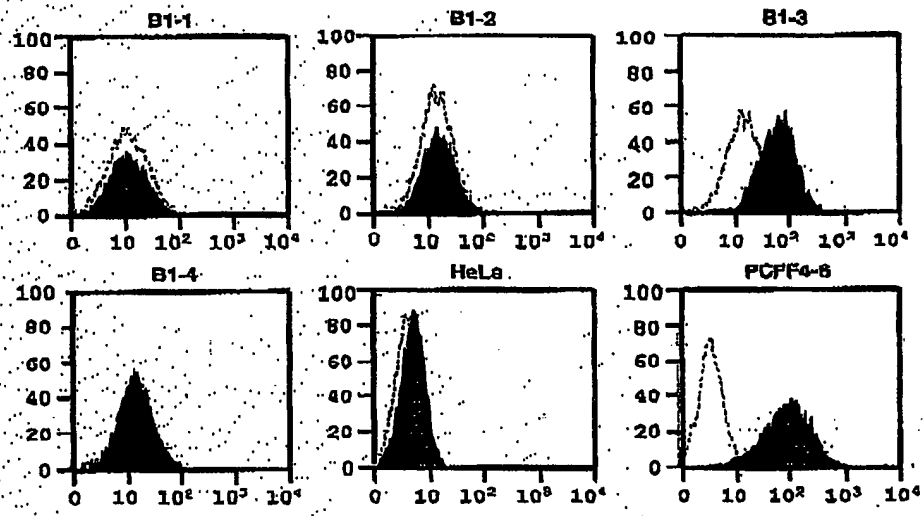


Fig. 1. Flow cytometry analysis of 680B1-1 to B1-4 cells with GS-IB4 lectin staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. Dotted line represents unstained cells analyzed by a fluorescence-activated cell sorter (Becton-Dickenson, Franklin, NJ). Shadow represents cells stained with fluorescein isothiocyanate–labeled GS-I-B4 lectin (EY Laboratories, Inc., San Mateo, CA). B1-1, B1-2, B1-3, and B1-4 are fetal fibroblasts derived from four day-39 fetuses. HeLa cells, a human cell line, were used as the negative control and PCFF4-6 cells, which were the parent cells for heterozygous and DKO of the α1,3GT gene, were used as the positive control.

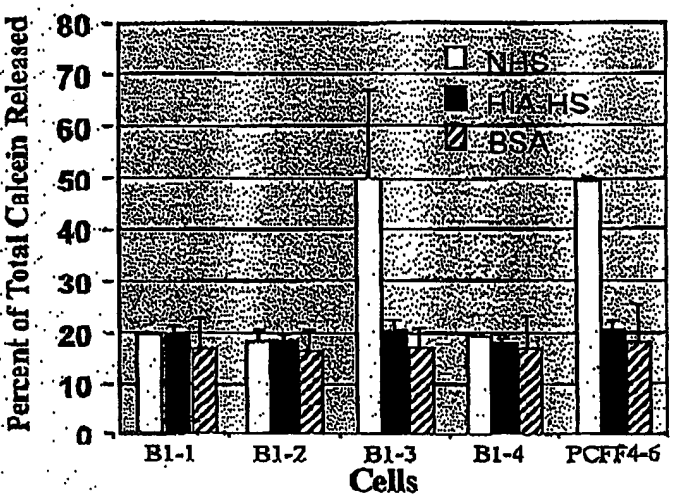


Fig. 2. Complement lysis assay for DKO fetal fibroblasts and wild-type pig cells. Results are the average of three individual assays. Open box represents NHS. Solid box represents heat-inactivated human serum (HIA-HS) as the negative control, and hatched box represents bovine serum albumin (BSA) as the reagent control. About 20% calcein release (no cells lysed) is the base value for the negative control (HIA-HS) and the reagent control (BSA). For quality control and reproducibility purposes, we did not use fresh human serum for the assay, which usually gives about 90% calcein release. About 50% calcein release (about 40% of cells lysed) from wild-type pig cells is typical with commercial serum (frozen and lyophilized) from Sigma.

B1-2, and B1-4 cells, but not in B1-3 cells or in the first knockout allele of all four cell lines. This T-to-G transversion in the α1,3GT coding region caused a single amino acid change from tyrosine to aspartic acid in the α1,3GT protein (Fig. 3). Although this mutation has not been observed in the inactivated α1,3GT gene of humans or higher primates (*14*), it is likely that the change of tyrosine, a hydrophobic amino acid, to aspartic acid, a hydrophilic amino acid, could disrupt α1,3GT function. Crystal structure analysis of bovine α1,3GT protein supports this speculation and shows that this tyrosine is at the center of the catalytic domain of bovine α1,3GT protein and is involved in uridine 5′-diphosphate–Gal binding (*15*, *16*).

To further confirm that the mutated cDNA cannot make functional α1,3GT protein, we cloned α1,3GT cDNAs from the nontargeted allele of B1-1 to B1-4 cells and wild-type pig cells into an expression vector and transfected them into human HeLa cells, which normally do not express α1,3GT protein. HeLa cells transfected with cDNA expression vectors from B1-1, B1-2, and B1-4 cells were negative for GS-IB4 lectin staining, indicating that the transfected pig cDNA from these cells did not make functional α1,3GT protein. In contrast, HeLa cells transfected with the cDNA from B1-3 cells and wild-type pig cells were positive with GS-IB4 staining. These results verified that the point mutation in cDNA from the second allele of the α1,3GT gene in B1-1, B1-2, and B1-4 cells resulted in synthesis of a defective α1,3GT protein. Although toxin A selection was repeated several times on 657A-III 1-6 cells, with or without pPL680 vector transfection, no additional toxin A–resistant colonies were detected.

We performed somatic cell nuclear transfer (cloning) with all three DKO cell lines as described in (*7*). We transferred cloned embryos into 16 estrus-synchronized recipient gilts. Ten initial pregnancies were established, only two of which went to term. Two pregnancies were lost before day 30, five were lost between day 30 and day 40, and one was lost by day 60. The first five female α1,3GT DKO piglets (761-1 to 761-5), cloned from 680B1-2 cells, were born on 25 July 2002. One piglet (761-1) died shortly after birth, and necropsy revealed an enlarged tongue and unusually large kidneys. We have observed this phenotype in a few other α1,3Gal-positive cloned pigs, and it appears to be a function of the cloning process (incomplete reprogramming) and not the α1,3GT gene knockout per se. The other four DKO piglets were of normal size and healthy. Aorta endothelial cells and muscle and tail fibroblasts isolated from the dead piglet (761-1) were negative with GS-IB4 lectin staining. FACS analysis of muscle fibroblasts from piglet 761-1 also showed a negative result for GS-IB4 binding. Neonatal tail fibroblasts isolated from the four healthy piglets, when analyzed by FACS with GS-IB4, were all negative (fig. S2). Tissue sections of liver, kidney, spleen, skin, intestine, muscle, brain, heart, pancreas, lung, aorta, tongue, umbilicus, and tail obtained from piglet 761-1 were all negative with GS-IB4 staining, indicating a complete lack of detectable cell surface α1,3Gal epitopes. The GS-IB4 staining results for liver sections from a newborn wild-type piglet and from piglet 761-1 are shown in fig. S3. Southern blot and sequencing analysis of DNA samples from all five piglets confirmed the targeted disruption of the first allele of the α1,3GT gene and the T-to-G point mutation in the second base of exon 9 in the second allele of the α1,3GT gene. It has been reported that α1,3GT DKO mice developed cataracts at 4 to 6 weeks of age (*17*). Physical examination of the four piglets at 7 weeks of age did not reveal any abnormalities or cataracts. We will continue to monitor the piglets for the presence of cataracts.

We performed an in vivo immunogenicity test with α1,3GT-knockout mice. We injected islet-like cell clusters (ICCs) isolated from the pancreas of piglet 761-1 intraperitoneally into α1,3GT knockout mice. We used ICCs from a neonatal wild-type piglet as a control. As shown in fig. S4, no increase in the titer of immunoglobulin M (IgM) to α1,3Gal was observed in α1,3GT knockout mice after injection with ICCs from the α1,3GT DKO piglet, in contrast to significant IgM titer increases observed in those mice injected with wild-type piglet ICCs. This result clearly demonstrates that the DKO piglet cells do not make any α1,3Gal epitopes.

Thus, we have successfully produced four α1,3GT-deficient piglets by a toxin A–mediated selection method. Although our intent was to knock out the second allele of the α1,3GT gene by homologous recombination, this did not occur. Instead, because we used this powerful selection method, which allows us to isolate any event that results in loss of α1,3GT activity, we discovered a mutation in the second allele of the α1,3GT gene. Had we used standard selection methods with puromycin or hygromycin, we would not have found the mutation. Although the rate of spontaneous mutation in the pig genome is very low [about $4 \times 10^{-8}$ for a spontaneous mutation per replication (*18*) in a mammalian gene similar in size to the α1,3GT gene], toxin A selection still enabled us to detect this crucial mutation. Clearly inactivation of the α1,3GT protein by this point mutation is a better outcome than by gene targeting with the pPL680 vector. It provides the opportunity to produce α1,3GT-deficient pigs without any antibiotic-resistance genes or other foreign DNA sequences, which should facilitate regulatory approval and, potentially, make a safer product for human use. It is certain that this point mutation will be maintained in the genome of these DKO pigs and their offspring, just as the few critical point mutations in the α1,3GT gene of humans and higher primates have been maintained over 20 million years (*14*). This genomic stability is not only due to the rarity of a reverse mutation event [about $5 \times 10^{-11}$ per replication (*18*) for mammals] but, more importantly, the strong selection pressure against α1,3Gal-positive cells by the presence of antibodies to α1,3Gal in α1,3Gal-negative animals. Our results have demonstrated that removal of α1,3Gal epitopes on pig cells did not preclude development in utero, even though pig cells express up to 500 times the number of α1,3Gal epitopes as do mouse cells (*4*, *19*). In addition, three consecutive rounds of cloning with rederived fetal cells did not appear to have a major detrimental effect on the overall development or health of the cloned pigs in this study. Analysis of tissues and organs from these α1,3GT DKO pigs in nonhuman primate models should provide clear indications of the involvement of α1,3Gal in HAR, AVR, and chronic rejection.

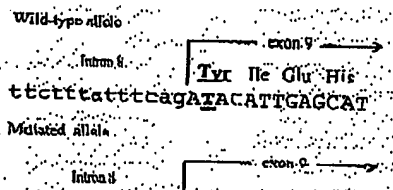


Fig. 3. Sequencing analysis of the α1,3GT gene from wild-type pig cells and DKO porcine fetal fibroblasts. Upper and lower alignment show nucleotide sequence of the α1,3GT intron 8–exon 9 boundary from wild-type pigs and the second allele of the DKO pig fetuses (B1-1, B1-2, and B1-4), respectively. Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate the nucleotide where the point mutation occurred. Amino acids deduced from the correspondent mutated and wild-type DNA sequence are underlined. No other mutations were found in the coding region of the α1,3GT gene from the second allele of the DKO pig fetuses in our genomic and reverse transcriptase–PCR libraries.

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12. pPL680 was made from three parts: a 1.8-kb PCR-

# Deletion of the α(1,3)galactosyl transferase (*GGTA1*) gene and the prion protein (*PrP*) gene in sheep

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Nuclear transfer offers a cell-based route for producing precise genetic modifications in a range of animal species. Using sheep, we report reproducible targeted gene deletion at two independent loci in fetal fibroblasts. Vital regions were deleted from the α(1,3)galactosyl transferase (*GGTA1*) gene, which may account for the hyperacute rejection of xenografted organs, and from the prion protein (*PrP*) gene, which is directly associated with spongiform encephalopathies in humans and animals. Reconstructed embryos were prepared using cultures of targeted or nontargeted donor cells. Eight pregnancies were maintained to term and four $PrP^{-/+}$ lambs were born. Although three of these perished soon after birth, one survived for 12 days. These data show that lambs carrying targeted gene deletions can be generated by nuclear transfer.



Gene targeting in embryonic stem (ES) cells is a powerful tool for modifying the genome of mice[1]. In other species, ES cells that contribute to the germline are not available, limiting widespread use of the technique. With the development of nuclear transfer in livestock species[2–4], genetically engineered somatic cells can be used to modify the genome. Previously, transgenic sheep expressing the human Factor IX gene in the mammary gland were produced by this route after random integration of the transgene into donor cell nuclei[4]. More recently, viable animals have been produced after gene targeting was used to precisely insert human α1-antitrypsin (AAT) sequences into the *COL1A1* locus[5], although the insertion site was specifically selected so as not to disrupt type 1 collagen protein function or expression. Targeted gene disruption is essential when complete deletion of gene function is required.

Animals potentially offer an alternative source of tissue for transplantation. A major barrier to successful xenotransplantation is presented by preformed antibodies that recognize the disaccharide galactose-α(1,3)-galactose, leading to hyperacute rejection[6]. Synthesis of galactose-α(1,3)-galactose is catalyzed by the enzyme α(1,3)galactosyl transferase, which is present in all organisms except catarrhines (Old World monkeys, apes, and humans). The hypothesis that deletion of this gene from the germline of donor species may eliminate a substantial component of hyperacute rejection needs to be tested in a large-animal model. Although the pig has been highlighted as the ideal choice for xenotransplantation, concerns have been raised about anatomical incompatibilities with humans[7] and the retroviral load of the porcine genome[8]. Sheep lacking α(1,3)galactosyl transferase could be used to determine the importance of galactose-α(1,3)-galactose in graft rejection, to develop immunosuppression regimes, and to provide tissues for xenotransplantation.

Prions, encoded by the *PrP* gene, are a novel form of infectious agent that cause spongiform encephalopathies in humans and animals[9]. Prions have assumed tremendous importance because of the bovine spongiform encephalopathy (BSE) epidemic and the concern that there has been cross-species transmission to humans, resulting in a new and highly lethal form of Creutzfeld–Jacob disease. Experiments with *PrP* gene knockout mice have shown that these animals do not replicate the prion gene and are resistant to scrapie[10,11]. Because sheep, and particularly cattle, have functional *PrP* genes and are used to produce biomedical products such as gelatin, collagen, and, increasingly, human proteins after genetic modification, it may be appropriate to produce prion-resistant populations.

We therefore selected the *GGTA1* and *PrP* genes as candidates for deletion from sheep. In addition, genetically engineered mice without one or the other of these genes show no gross deleterious effects[12–14], indicating that they would be appropriate targets to develop gene disruption technology in livestock. Here we report use of nuclear transfer to produce sheep that have targeted gene deletions.

## Results and discussion

The ovine *PrP* gene has previously been cloned and characterized. Three exons span 21 kilobases of genomic DNA, with the 770 base pair coding region contained entirely within the final exon[15]. Comparable data for the *GGTA1* gene were not available, although the coding sequence was known for other species[16,17]. Using primers that functioned across species in a reverse transcriptase–polymerase chain reaction (RT-PCR), we isolated an 1,110 base pair ovine *GGTA1* complementary DNA (cDNA), which showed 83% and 95% homology to murine and bovine sequences, respectively. A 193 base pair 5′-untranslated region was extended by rapid amplification of cDNA ends (RACE) PCR, although it appears to be truncated compared with the corresponding region in the mouse gene.

To generate targeting vectors, we used *GGTA1* or *PrP* DNA probes to screen a genomic library prepared from tissue culture cells derived from a day 35 Black Welsh fetus. The coding exons of the *GGTA1* gene span ~20 kilobases of genomic DNA and were designated 4 to 9 (Fig. 1), because translation initiation occurs in exon 4 of the well-characterized mouse gene[16]. The *PrP*-hybridizing phage were analyzed and had the same sequence and restriction pattern as in the published data[15].

The *GGTA1* and *PrP* genes are expressed in fetal fibroblasts (data not shown), permitting use of the promoter trap targeting strategy[18]. In the vectors constructed, the neomycin phosphotransferase (*neo*) gene was

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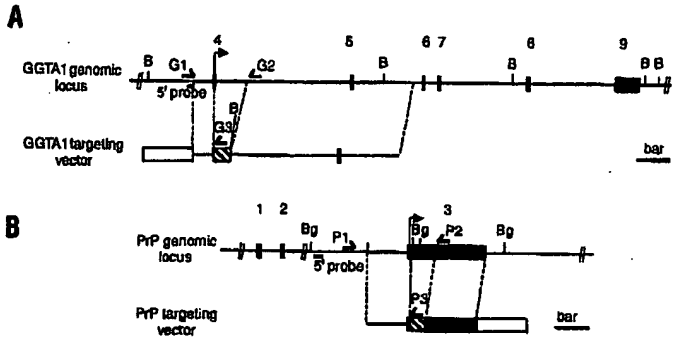


**Figure 1.** Organization of the genomic loci of ovine (A) *GGTA1* or (B) *PrP* genes and the promoterless targeting vectors used for disruption. Numbering of the exons in *GGTA1* is based on the mouse; translation initiates in exon 4 and terminates in exon 9. Targeting deletes exon 4 and 1.4 kb of intron 4, and a *BamHI* site (labeled B) is inserted. The coding sequence of *PrP* is entirely within exon 3; targeting deletes this region and two *BglI* sites (labeled Bg). Arrows indicate translation initiation sites. Black boxes represent exons, hatched boxes represent *neo*-pA sequence, and open box represents pBlueScript sequence. Location of PCR primers (*GGTA1* uses G1/2 and G1/3; *PrP* uses P1/2 and P1/3) and the 5′ external probes for Southern blot analysis are shown. Scale bar represents 2 kb.

placed directly adjacent to the initiation codon of the target genes (Fig. 1). The *PrP* targeting vector does not delete the splice acceptor site of exon 3, a component of the gene that must be retained to generate knockout mice that are clinically healthy and do not have a grossly aberrant phenotype[14]. Linearized *GGTA1* or *PrP* vectors (10 μg) were transfected into early-passage BW6F2 karotypically normal male (54XY) cells. After 12 days of G418 selection, 877 and 533 colonies had grown in the *GGTA1* and *PrP* experiments, respectively (Table 1).

Initially, we used two independent PCR reactions to detect targeting events for each construct. Using this strategy, we demonstrated that 1.1% (10) or 10.3% (55) of the *GGTA1* or *PrP* BW6F2 neomycin-resistant ($neo^R$) colonies contained correctly targeted cells (Table 1). However, in terms of selecting a clonal targeted population with a stable karyotype that could be expanded for use in several nuclear transfer (NT) experiments, only one colony ($PrP^{+/+}$, termed YH6) was suitable (Table 1; Fig. 2B, lane 1). Many targeted colonies also contained nontargeted cells, as indicated by the greater intensity of the PCR band from the nontargeted allele compared with that of the targeted allele. More importantly, a substantial number of colonies (4/5 *PrP* and 8/8 *GGTA1*) with only targeted cells senesced before they could be prepared for nuclear transfer (Table 1). The high attrition rate of targeted clonal populations suitable for nuclear transfer (Table 1) represents one of the major hurdles of gene targeting in primary somatic cells.

Targeting experiments at the *GGTA1* locus were continued using a different primary cell culture, 7G65F4, isolated from a Finn Dorset fetus. These cells were in culture for 6 days before electroporation, compared with ~14 days for the BW6F2 cells used before. Targeting events were detected at a frequency of 6.2% (35 of 568). Ultimately, two *GGTA1*-targeted colonies (3C6 and 5E1) suitable for nuclear transfer were isolated (Table 1; Fig. 2A, lanes 1 and 4).

We have shown targeting frequencies in $neo^R$ clones of 1.1 and 6.2% for the *GGTA1* locus and of 10.3% for the *PrP* locus. These are upper estimates, as the data include a substantial proportion of mixed clones, but correspond to an overall targeting frequency of 1–10 per $10^6$ cells. Recently McCreath and colleagues[5] reported targeting efficiencies of 7.1, 13.8, and 65.7% in the ovine *COL1A1* locus in Poll Dorset fetal fibroblasts. The high average efficiency in these experiments may be attributable to high endogenous expression or intrinsic recombinogenic activity at this locus. Alternatively, the vector used by these workers had contiguous regions of homology with the chromosomal locus and did not delete any of the *COL1A1* gene. By contrast, to ensure effective disruption of the *GGTA1* and *PrP* genes, we deleted endogenous coding sequence with *neo*-polyA sequence using noncontiguous regions of homology.

Targeted (3C6, 5E1, or YH6) and control cells (4H2, with a random integration of the *GGTA1* targeting vector: Fig. 2A, lane 7; 7G65F4, nontransfected parental line) were prepared for nuclear transfer by culturing in low- (0.5%) serum medium for three to five days. Donor cells were fused to enucleated Poll Dorset oocytes, as described[2]. A total of 120 morulae or blastocysts were transferred to 78 Finn Dorset final recipients, which produced 39 pregnancies at day 35. The oldest *GGTA1*-targeted fetuses died *in utero* at 118 and 130 days (term 148 days). Eight pregnancies were maintained to term (two 7G65F4, one 4H2, five YH6), resulting in four live births derived from the *PrP*-deleted line, YH6. Three of these lambs perished soon after birth. One lived for 12 days (Table 2; Fig. 3) but was euthanized after developing dyspnea due to pulmonary hypertension and right-sided heart failure, common abnormalities in cloned sheep.

The high incidence of mortality reported here may indicate that genetic modification or prolonged culture is detrimental to development. Although comparison of the developmental stages revealed similar efficiencies of progression from targeted cells, nontargeted cells with random integration, and untransfected cultures (blastocyst, 10–31%; day 35, 3.3–6.7%; day 60, 0–4.4%, referenced to embryos transferred or cultured; Table 2), we observed a high incidence of mortality at and soon after birth. This contrasts with other studies using unmodified, early-passage sheep cells[2–4]. However, it is consistent with a recent report[5] of gene insertion in sheep; although two targeted animals survived beyond three months, there was a high incidence of perinatal and postnatal mortality. Thus prolonged culture, in combination with the stringent selection required for somatic gene targeting, may produce cell lines that are less competent at producing viable clones.

When possible, autopsies were performed. The range of abnormalities found was consistent among the different groups. The predominant findings were hydroallantois, distention of the liver caused by congestion (suggestive of cardiac insufficiency), insufficient placentation indicated by reduced numbers and size of cotyledons, and kidney dysplasia manifested by enlarged renal pelvis with narrowed cortex and medulla. All these defects have been described in other nuclear transfer experiments with nontrans-

**Table 1. Efficiency of gene targeting in ovine somatic cells**

| Parental primary culture | Target gene | G418-resistant colonies | Total targeting events detected[a] | Mixed colonies[b] | Senesced[c] | Unstable[d] karyotype | Targeted colonies suitable for NT |
|---|---|---|---|---|---|---|---|
| BW6F2 | *GGTA1* | 877 | 10 | 2 | 8 | 0 | 0 |
| BW6F2 | *PrP* | 533 | 55 | 50 | 4 | 0 | 1 (YH6) |
| 7G65F4 | *GGTA1* | 568 | 35 | 17 | 15 | 1 | 2 (3C6, 5E1) |

[a]Total number of targeting events detected by the initial PCR screens.
[b]Colonies were scored as mixed when the amplified band from the nontargeted locus was more intense than the targeted locus in the second PCR screen.
[c]Colonies were scored as senesced when cell numbers could not be seen to increase after seven days.
[d]The normal karyotype of these cells was 54XY.

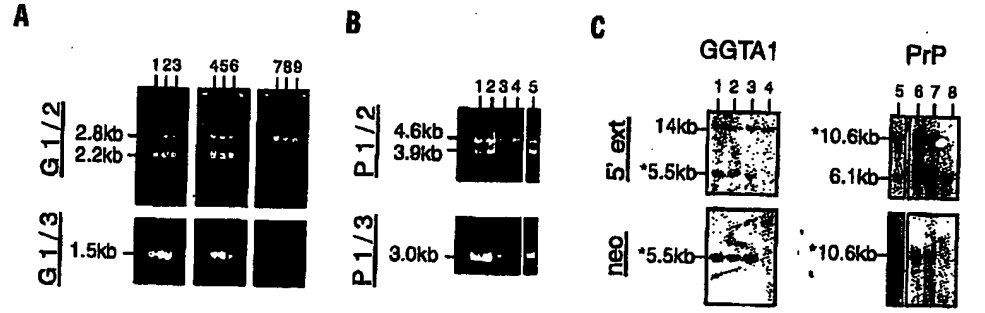


**Figure 2.** Targeted mutations are retained through development. DNA was isolated from cells before nuclear transfer or from derived fetuses, then analyzed by PCR and Southern blot. Samples with a targeted allele are indicated by an asterisk (*). See Figure 1 for location of primers and probes. (A) *GGTA1* PCR. Lanes 1, 2, and 3 show 3C6* cells, and fetuses at day 85* and day 118*. Lanes 4, 5, and 6 show 5E1* cells, and fetuses at day 49* and day 49*. Lanes 7, 8, and 9 show 4H2 cells, and fetuses at day 49 and day 148. (B) *PrP* PCR. Lane 4 shows nontargeted parental cells. Lane 1 shows YH6* cells. Lanes 2 and 3 show lambs carried to term*. Lane 5 shows the targeted lamb that survived to 12 days*. (C) Southern blot analysis. *GGTA1* samples were digested with *Bam*HI. The targeted allele hybridizes with the 5' and neo probes; lanes 1, 2, and 3 show samples from fetuses at day 118*, day 49*, and day 49*. Lane 4 shows a nontargeted sample. *PrP* samples were digested with *Bgl*I. Lane 5 shows the targeted lamb that survived to 12 days*. Lanes 6 and 7 show samples from fetuses at term*. Lane 8 shows a nontargeted sample.

**Figure 3.** *PrP*$^{-/-}$ lamb photographed at six days postpartum.

fected cells[2,5,19,20]. We did not expect abnormal phenotypes as a direct result of the gene disruption because we modified only one allele at a dominant locus. Furthermore, null mice for *GGTA1* or *PrP* are healthy[12–14].

Tissue was recovered from fetuses and lambs for both PCR and Southern blot analysis. Data are shown for fetuses ranging from day 49 to 148 (term) of pregnancy. The two PCR screens for each locus revealed patterns consistent with targeting (Fig. 2) in all the samples that were recovered. In Southern blot analyses, both 5' (external) and neo coding sequence (internal) probes hybridized to restriction fragments of the correct size. The location of probes and restriction sites is shown in Figure 1; representative Southern blots are shown in Figure 2. These data show that lambs carrying targeted gene deletions can be generated by nuclear transfer.

Our results, together with the recent report of sequence insertion at the ovine *COL1A1* locus[5], indicate that targeted homologous recombination has been demonstrated at three independent loci in cells derived from different breeds of sheep. This suggests that the technology can be used to disrupt many different genes in the ovine genome. We found, however, that the number of targeted clones suitable for nuclear transfer was low. A major barrier was that many of the clonal populations reached proliferative senescence. The bulk populations of the primary cultures we used divide ~100 times before senescing, a large excess compared to the estimated 45 doublings required for targeting and preparation for nuclear transfer[21]. A likely explanation is that there is considerable heterogeneity of life span in the culture, with many of the selected colonies having a life span considerably shorter than 100 doublings.

The death of the targeted fetuses and lambs emphasizes the need to improve the efficiency of the technology. Once this is achieved, effective ablation of gene function will usually require both alleles to be disrupted. Given the limited proliferative capacity of cells currently used in nuclear transfer, achieving this from a single clonal population will be difficult. Alternatively, conventional breeding could be used with animals surviving to reproductive maturity. However, this would take a minimum of 18 months in sheep, even if the modification were introduced simultaneously into male and female cells and the cloned animals interbred. A different approach would be to clone by nuclear transfer from the cells in which the first allele has been targeted, re-isolate cell lines from the cloned fetal material, and then target the second locus in these cells[22,23]. Ultimately, however, the fastest route to multiple genetic changes would be to extend the window to achieve targeting, either by increasing the overall efficiency of targeting or by using cells with an extended life span that still retain their totipotency for nuclear transfer.

**Table 2. Nuclear transfer from gene-targeted primary cells[a]**

| Stage of nuclear transfer | Cells used for nuclear transfer | | | | |
|---|---|---|---|---|---|
| | 3C6 | 5E1 | 4H2 | 7G65F4 | YH6 |
| Embryos transferred into temporary recipients[b] (*in vitro* cultured) | 87 (25) | 0 (30) | 92 (31) | 55 (71) | 273 (181) |
| Embryos recovered from temporary recipients | 85 | – | 62 | 55 | 214 |
| Morula or blastocyst[b]: *in vivo* (*in vitro*) | 18 (7) | 0 (3) | 19 (8) | 12 (27) | 44 (3) |
| Embryos transferred to final recipients | 18 | 3 | 23 | 33 | 43 |
| Final recipients | 12 | 3 | 17 | 18 | 28 |
| Fetuses at day 35 | 7 | 2 | 4 | 8 | 18 |
| Fetuses at day 60 | 5 | 0 | 2 | 5 | 8 |
| Lambs at birth: live (dead) | 0 | 0 | 0 (1) | 0 (2) | 3 (1) |
| Lambs alive at one week | 0 | 0 | 0 | 0 | 1 |

[a]Data are shown for various cultures: 3C6 and 5E1 (*GGTA1* correctly targeted), 4H2 (randomly integrated *GGTA1* targeting vector), and 7G65F4 (untransfected cells) were of Finn Dorset origin; YH6 (*PrP* correctly targeted) was of Black Welsh origin. Poll Dorset oocytes were used as recipient cytoplasts throughout.
[b]Reconstructed embryos were transferred to temporary recipients, unless the number of oocytes recovered was low or fusion could not be seen and *in vitro* culture (additional embryos shown in parentheses) was adopted.

## Experimental protocol

**Isolation, culture, and transfection of primary fibroblasts.** Black Welsh (BW6F2) or Finn Dorset (7G65F4) fibroblasts were recovered from day 35 fetuses as described[2]. Cells were cultured in BHK21 medium (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids (Life Technologies, Rockville, MD), and 10% FCS (Globe Farm, Gilford, Surrey, UK) in a humidified environment with 5% $CO_2$. Linearized targeting construct (10 μg) was electroporated to passage one 7G65F4 (125 μF/350 V, *GGTA1*) or passage six BW6F2 (250 μF/400 V, *PrP*) cells ($5 \times 10^6$), which were then seeded in 96-well plates ($2.5 \times 10^3$ cells/well). G418 selection (400 μg/ml) was applied after 24 h. At subconfluence, resistant colonies were replica plated to two 96-well plates for DNA analysis or cryopreservation.

**Targeting constructs.** Promoterless vectors, with *neo-pA* sequence (Stratagene, La Jolla, CA) adjacent to the endogenous gene start codon, were used to target the *GGTA1* and *PrP* loci. The *GGTA1* vector was constructed by amplifying a truncated left arm (300 bp; using primers 199001, 5′-ACGTGGCTCCAAGAATTCTCCAGGCAAGAGTACTGG-3′ and 199006, 5′-CATCTTGTTCAATGGCCGATCCCATTATTTTCTCCTGGGAAAAGAAAAG-3′, with tail complementary to the start of neo coding sequence) and neo-polyA sequence (using primers 199005, 5′-CTTTTCTTTTCCCAGGAGAAAATAATGGGATCGGCCATTGAACAAGATG-3′, with tail complementary to left arm, and 199004, 5′-CAGGTCGACGGATCCGAACAAAC-3′). These fragments were used to prime from each other to give a 1.2 kb fusion product. This was ligated to intron 3 sequence (1 kb *Eco*RV-*Eco*RI fragment), to extend the left arm, and to ~9 kb (*Eco*RV partial digest–*Not*I) of 3′ sequence to create the right arm.

The *PrP* vector was constructed by amplifying the left arm (2.4 kb; using primers prp6F, 5′-CCGAGCTCGCCAATTTCATGGCTGCAGTCACC-3′; and prp7R, 5′-CGATCCCATGATGACTTCTCTGCAAAATAAAG-3′, with tail complementary to the start of neo coding sequence) and neo-polyA sequence (using primers prp10F, 5′-GAGAAGTCATCATGGGATCGGCCATTGAACA-3′, with tail complementary to left arm; and prp8R, 5′-TGCAGGTCGACGGATCCGAA-3′). These fragments were used to prime from each other to give a 3.3 kb fusion product, which was ligated to a 3 kb *Kpn*I fragment to complete the vector.

The *GGTA1 or PrP* vectors were linearized with *Not*I or *Sac*I, respectively, before electroporation.

**DNA analysis.** Drug-resistant colonies were screened for targeting events by PCR. DNA was isolated in 96-well plates by overnight lysis (50 mM Tris, pH 8, 20 mM ethylenediamine tetraacetate, 100 mM NaCl, 0.3% sodium dodecyl sulfate, 10 mg/ml proteinase K), then isopropanol precipitated, and pellets were resuspended in 50 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). Amplification was performed using Roche Expand HiFi kit, with 1 μl DNA template. Primer locations are indicated in Figure 2: G1 (5′-CAGCTGTGTGGGTATGGGAGGG-3′); G2 (5′-CTGAACTGAATGTTTATCCAGGCCATC–3′); G3 / P3 (5′-AGCCGATTGTCTGTTGTGCCCAGTCAT–3′); PR1 (5′-TTCAGTCGCTCTGTTGTGTC CCA-3′); P2 (5′-AGCATCCCTC CTGCCTTCAG TTCTTC-3′). Cycling conditions for *GGTA1* were 94°C, 2 min/94°C, 30 s / 65°C, 30 s / 68°C, 2.5 min (10 cycles); 94°C, 30 s / 65°C, 30 s / 68°C, 2.5 min + 5 s per cycle (20 cycles); 68°C, 7 min. For *PrP* the elongation phase was increased to 4 min. Products were analyzed by agarose gel electrophoresis.

For Southern blot analysis, genomic DNA was digested with *Bam*HI or *Bgl*I (*GGTA1* or *PrP*, respectively) and blotted to Ambion bright star membrane according to manufacturer's instructions. Diagnostic bands were detected using Ultrahyb (Ambion, Austin, TX) with DNA probes corresponding to *neo* sequence (Stratagene), *GGTA1* 5′ probe (a 100 bp fragment was produced by PCR using forward [CAGCTGTGTGGGTATGGGAGGG] and reverse [CTAACTACGTGCTCCGCCGTTCA] primers) or *PrP* 5′ probe (corresponding to 16,701–17,151 bp of accession no. U67922, Entrez, NCBI).

**Nuclear transfer.** Somatic cell nuclear transfer was based on the method of Wilmut[2]. Oocytes were collected from superovulated Poll Dorset ewes in PBS with 1% FCS and transferred immediately to calcium-free HEPES-buffered synthetic oviduct fluid[19] (SOF) for removal of cumulus and enucleation. If necessary, cumulus was removed by pipetting in 600 IU/ml hyaluronidase. Oocytes were exposed to 5 μg/ml Hoechst 33248 and 7.5 μg/ml cytochalasin B. Sheep fetal fibroblasts were cultured for three to five days in serum-deficient medium (0.5% FCS) before use as karyoplast donors. Simultaneous fusion of donor cells and recipient oocytes, and activation of the recipient oocytes, was achieved by three consecutive 80 μs pulses of 1.25 kV/cm² in 0.3 M mannitol, 0.1 $MgCl_2$, and 0.05 mM $CaCl_2$. Reconstructed embryos were incubated for six days (*in vitro* culture) or overnight (*in vivo* culture) in SOF solution supplemented with BSA in an atmosphere consisting of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ at 38°C. For *in vivo* culture, following the overnight culture, embryos were embedded in 1% agar chips in PBS and transferred into the ligated oviduct of an estrus-synchronized recipient ewe for an additional six days. Morula and blastocyst stage embryos were recovered seven days post-activation to the uteri of estrus-synchronized ewes (one to two embryos/recipient). Pregnancies were monitored using subcutaneous ultrasound scanning.

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# Production of $\alpha$-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations

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**Hyperacute rejection of porcine organs by old world primate recipients is mediated through preformed antibodies against galactosyl-$\alpha$-1,3-galactose (Gal$\alpha$-1,3-Gal) epitopes expressed on the pig cell surface. Previously, we generated inbred miniature swine with a null allele of the $\alpha$-1,3-galactosyltransferase locus (*GGTA1*) by nuclear transfer (NT) with gene-targeted fibroblasts. To expedite the generation of *GGTA1* null pigs, we selected spontaneous null mutant cells from fibroblast cultures of heterozygous animals for use in another round of NT. An unexpectedly high rate of spontaneous loss of *GGTA1* function was observed, with the vast majority of null cells resulting from loss of the WT allele. Healthy piglets, hemizygous and homozygous for the gene-targeted allele, were produced by NT by using fibroblasts that had undergone deletional and crossover/gene conversion events, respectively. Aside from loss of Gal$\alpha$-1,3-Gal epitopes, there were no obvious phenotypic differences between these null piglets and WT piglets from the same inbred lines. In fact, congenital abnormalities observed in the heterozygous NT animals did not reappear in the serially produced null animals.**

Antibodies against galactosyl-$\alpha$-1,3-galactose (Gal$\alpha$-1,3-Gal) residues on cell surface glycoproteins of pig cells mediate hyperacute rejection of porcine organs in primate model recipients and are the most immediate barrier to successful clinical xenotransplantation (1, 2). High levels of preformed "natural" antibodies against the Gal$\alpha$-1,3-Gal epitope are found in humans and old world primates, following evolutionary loss of the corresponding galactosyltransferase activity (encoded by *GGTA1*) (3). The presence of these antibodies, along with the high density of Gal$\alpha$-1,3-Gal residues on most pig cells (4), suggests that elimination of *GGTA1* function would provide a practical means of overcoming both hyperacute rejection and subsequent acute or chronic tissue damage associated with antibody binding to this epitope.

The lack of *GGTA1* function in humans and old world primates, along with the viability of *GGTA1* knockout mice produced with embryonic stem cell technology (5, 6), suggested that a knockout strategy might be biologically feasible in pigs. The cloning of sheep (7) and subsequently pigs (8–10) by nuclear transfer with somatic cells has made attempts to knockout the *GGTA1* locus in pigs technically feasible.

We have previously reported the generation of *GGTA1* heterozygous inbred miniature swine using nuclear transfer with gene-targeted fibroblasts (11). Starting with heterozygous fibroblasts from such animals, we now report the isolation of *GGTA1* null cells with spontaneous loss of the WT allele. The rate of loss of heterozygosity (LOH) was several orders of magnitude greater than typically expected, an observation that may be related to the inbred background of the heterozygous animals. LOH resulted in some cases from deletion of the WT allele and in others from either somatic crossing over or gene conversion. Similarly high rates of somatic recombination, subject to modulation by genetic background and chromosomal structure, have been reported in the mouse (12). Generation of healthy piglets with both hemizygous and homozygous *GGTA1* null cells demonstrates that such somatic LOH mutations can be introduced into large animal genomes by nuclear transfer, in a manner analogous to that using murine embryonic stem cell chimeras (13).

## Methods

***GGTA1* Heterozygous Cell Lines.** ***355-F1.*** Fetus 355-F1 was generated by nuclear transfer from cultured ear fibroblasts of pig O212-2, a *GGTA1* heterozygote in which one allele has been inactivated by homologous recombination with vector pGalGTΔS-Neo (11). Cells were isolated at day 33 of gestation by digestion with collagenase/thermolysin (Blendzyme 3, Roche Diagnostics, Indianapolis, IN) and cultured in Ham's Nutrient Mixture F10 (Invitrogen Life Technologies, Baltimore) containing 20% FBS. ***PL556.*** Piglet PL556 was derived by nuclear transfer. The donor cell clone, F501-F4, was produced by targeting of fetal fibroblasts from WT fetus F501 with vector pGalGTΔS-Neo, as described (11). PL556 cells were cultured in high glucose DMEM (Invitrogen Life Technologies, Baltimore) containing 10% FBS and 0.1 mM 2-mercaptoethanol.

Cell culture for all work reported here, beginning with tissue acquisition, was done in the absence of G418.

**Nuclear Transfer (NT).** For generation of piglets from clonal null cell lines, oocytes from sow ovaries were purchased (BoMed, Madison, WI), and NT was performed as described (11). The surviving embryos, possessing an intact plasma membrane, were selected for transfer into recipients after culture for 18–22 h. Potential domestic recipients were heat checked twice a day. Depending upon the exact time of estrus, 100–180 NT-derived embryos were transferred into recipient oviducts 5–17 h or 20–36 h after the onset of estrus for day 0 and day 1 recipients, respectively.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: NAb, natural antibody; LOH, loss of heterozygosity; NT, nuclear transfer; Gal$\alpha$-1,3-Gal, galactosyl-$\alpha$-1,3-galactose; LDH, lactate dehydrogenase; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt].

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For NT with nonclonal null cell-enriched populations, oocyte maturation, NT, embryo culture, and embryo transfer into recipient females were performed as described (8), except that female recipients were selected that exhibited first standing estrus within 12 h of cybrid activation.

**Baboon Natural Antibody.** Anti-Gal$\alpha$-1,3-Gal antibodies from naive baboon plasma (natural antibody, NAb) were affinity-purified by absorption to Gal$\alpha$-1,3-Gal LB-VI matrix columns (14) (Alberta Research Council, Alberta, Canada). The bound NAb was eluted from the column in 0.25% acetic acid, neutralized, and dialyzed against PBS before concentration and sterile filtration.

**Null Cell Selection.** To enrich for *GGTA1* null cells in fibroblast cultures, 355-F1 cells and PL556 cells were cultured in F10 medium containing 20% FBS and 20 $\mu$g/ml gentamycin on collagen I-coated dishes at 5% $CO_2$, 3% $O_2$, and 37°C. The above cell lines were treated in suspension at $2 \times 10^6$ cells/ml in 100 $\mu$g/ml affinity-purified baboon NAb in media for 30 min at room temperature with mixing. After washing, cells were then treated with 12.5% baby rabbit complement (Pel-Freez Biologicals) containing DNase I (10 $\mu$g/ml) in media for 45 min at room temperature with mixing. Surviving cells were counted and plated in bulk culture and expanded for subsequent treatments. This selection was repeated three times for 355-F1 cells and twice for PL556 cells. Selections were performed every 7–10 days, with the fourth selection of 355-F1 performed 3 days after the third selection. Before each NAb/complement selection, cells were analyzed for the presence of Gal$\alpha$-1,3-Gal epitopes with FITC-conjugated BS-I-$B_4$.

For clonal selection, 355-F1 cells were treated twice in suspension as above with 50 $\mu$g/ml NAb and 12.5% complement, with 4 days between treatments. After the second treatment, cells were plated at 5 and 10 cells/well in collagen I coated 96-well plates. *In situ* treatments with 100–500 $\mu$g/ml NAb for 1 h at 37°C and 12.5% complement for 1 h at 37°C were performed every other day for treatments 3–5. Wells containing patches of cells covering >15% of the well were transferred to a 48-well plate and treated the following day *in situ* with 500 $\mu$g/ml NAb and complement. Cells were passaged for molecular analysis, BS-I-$B_4$ analysis, and freezing.

**Flow Cytometry Analysis.** Gal$\alpha$-1,3-Gal epitope expression was analyzed with FITC-conjugated BS-I-$B_4$ lectin (Sigma). Unfixed cells were stained for 5 min at 37°C in 4 $\mu$g/ml lectin, washed, and then resuspended in buffer containing propidium iodide (PI). Fluorescence data were collected on a Becton Dickinson FACScan, and analysis of PI excluding cells was performed by using CELLQUEST flow cytometry software (BD Immunocytometry Systems).

**Quantitative Southern Blots.** Genomic DNA was digested with *Afl*III, which generates a 1,280-bp fragment of the WT *GGTA1* allele (sites at bp 9 and bp 1,289 of GenBank accession number AF221517) and a 2,330-bp fragment of the pGalGTΔS-neo targeted allele (11). Southern blots were simultaneously probed with $^{32}$P-labeled RNA transcripts from the exon 9 portion of this fragment (bp 776–891 of GenBank accession number AF221517) and a portion of the porcine DQ-$\beta$ locus (bp 901-1015 of GenBank accession number M31497). Phosphorscreen autoradiography was performed on a STORM 820 Optical Scanner, and area quantitation was done with IMAGEQUANT 5.2 software (Molecular Dynamics).

**Microsatellite Analysis.** PCR was performed by using WellRED-labeled primers, and the reactions were analyzed on a CEQ2000 sequence analyzer (Beckman Coulter). Markers Sw2518 and Sw1430 map to porcine chromosome 1 at ≈67 cM and 58 cM, respectively; combined radiation hybrid and genetic data place the *GGTA1* locus at ≈115–122 cM (www.genome.iastate.edu/pig). Heterozygosity of marker Sw2518 in fetus 355-F1 and Sw1430 in piglet PL556 was confirmed by segregation of alleles within the respective inbred miniature swine lineages.

**Complement-Mediated Lysis.** Lysis [lactate dehydrogenase (LDH) release] and metabolism {conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]} were measured by using the Cytotox 96 NonRadioactive Cytotoxicity and CellTiter 96 Aqueous One Solution Cell Proliferation assays (Promega), respectively. Human serum was prepared by heat inactivation of a pool from 10 untyped individuals. Preparation of affinity-purified baboon NAb was as described above. Baby rabbit complement was obtained from Pel-Freez Biologicals. Normal human dermal fibroblasts (Cambrex Bioscience, Walkersville, MD) served as Gal$\alpha$-1,3-Gal negative cell controls.

Two days after plating in triplicate wells, subconfluent fibroblasts were incubated in medium (Ham's Nutrient Mixture F10, 5% FBS) containing NAb or human serum for 30 min at 37°C. The cells were washed twice with Ham's Nutrient Mixture F10 and then incubated for 60 min at 37°C in the above medium containing 12.5% rabbit complement. Medium from this incubation was assayed for LDH release. Remaining cells were incubated in medium containing 16.5% MTS for 2.5–3 h. After incubation, 25 $\mu$l of 10% SDS was added to all wells, and the medium was assayed for MTS conversion. Samples from triplicate "no cell" controls for each treatment condition were used to correct for assay background. For MTS assays, corrected average values are expressed as the percentage of corrected absorbance without NAb or serum for each line. For LDH assays, corrected average values are expressed as the percentage of corrected average absorbance after detergent lysis for each line. LDH release using anti-pig pan tissue mAb 1030h-1-19 (BD Biosciences PharMingen) was used as a positive control for porcine cell lysis, with similar titrations obtained for all three porcine fibroblast lines (data not shown).

## Results

**Selection of *GGTA1* Null Lines and Clones from Fetal and Neonatal Heterozygous Cell Lines.** Two sources of heterozygously targeted primary fibroblasts were chosen for selection of *GGTA1* null cells (Fig. 1). Fibroblasts from fetus 355-F1 were isolated at 33 days gestation after NT using ear fibroblasts from *GGTA1* heterozygous gilt O212-2. O212-2 was itself generated by NT by using gene-targeted fetal fibroblast clone F7-H6 as described (11). Similarly, ear fibroblasts from male *GGTA1* heterozygous neonate PL556 were isolated after NT by using gene-targeted fibroblast clone F501-F4.

Approximately $1.5 \times 10^7$ cells from established cultures of both sources were depleted of Gal$\alpha$-1,3-Gal epitope-bearing cells by lysis with affinity-purified baboon antibodies against the epitope in combination with complement. Enrichment for Gal$\alpha$-1,3-Gal-negative cells was monitored by flow cytometry analysis with FITC-labeled BS-I-$B_4$ lectin, which binds specifically to Gal$\alpha$-1,3-Gal epitopes. Initial depletions resulted in recovery of ≈0.1% of the cells, 7–15% of which were Gal$\alpha$-1,3-Gal negative. After 3–4 rounds of selection and expansion, these populations were essentially devoid of BS-I-$B_4$ binding cells (Fig. 2). DNA prepared from the null selected populations was analyzed by PCR that distinguishes WT and targeted *GGTA1* alleles. The absence of a readily detectable WT band indicated that the vast majority of these cells had undergone at least partial loss of the WT allele (Fig. 2). This loss of heterozygosity in the *GGTA1* null cells is compatible with chromosome loss and reduplication, interstitial deletion, or somatic recombination.

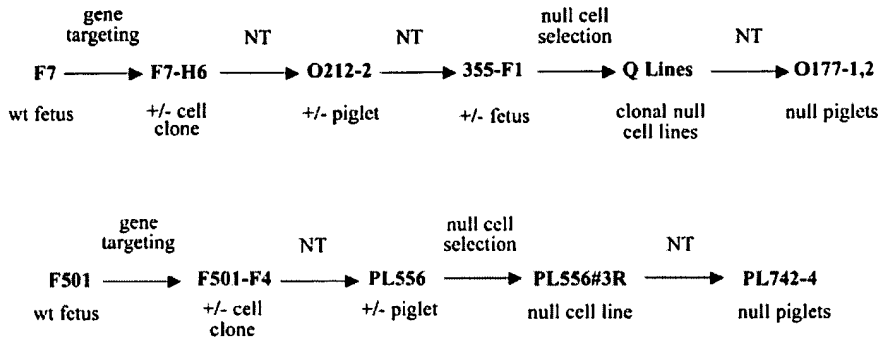


**Fig. 1.** Derivation of *GGTA1* null pigs.

Clonal null lines were isolated from a 355-F1 cell population that had been previously enriched for null cells by NAb/complement depletion. The recovery rate of null clones from the initial heterozygous lines was $\approx 10^{-4}$ in two separate trials. Microsatellite analysis of 28 clones with centromere proximal marker Sw2518 (heterozygous in 355-F1) revealed that all clones remained heterozygous at this locus, indicating that chromosome loss and reduplication was not the mechanism for loss of heterozygosity (LOH) in these clones. DNA samples from four of these clonal lines (Q2, Q9, Q32, and Q37), along with the 355-F1 and PL556 null selected populations, were analyzed by quantitative Southern blotting by using an exon 9 *GGTA1* probe present in both the targeted and WT alleles. A probe for the nonlinked porcine SLA *DQβ* gene served as a diploid copy number control (Fig. 3). In both null selected populations and all four clones, only a targeted length *GGTA1* allele was detected. In two null clones (Q2 and Q32) a *DQβ/GGTA1* signal ratio of $\approx$2:1 was obtained, demonstrating deletion of at least a portion of the WT *GGTA1* allele present in 355-F1. In comparison, clones Q9 and Q37 had *DQB/GGTA1* signal ratios of $\approx$1:1, indicating that loss of heterozygosity in these clones occurred through either somatic crossing over or gene conversion. Efforts to distinguish between these mechanisms by microsatellite analysis were unsuccessful because no heterozygous markers distal to Sw2185 were identified in fetus 355-F1 from among 24 tested. Sequencing of the junctions between exon 9 and the G418 selection cassette in clones Q9 and Q32 revealed no evidence of nucleotide heterozygosities (data not shown).

**Generation of *GGTA1* Null Pigs by NT.** NT was performed by using the four clonal fetal cell lines characterized by Southern analysis (Q2, Q9, Q32, and Q37), as well as the null cell population selected from neonate PL556. Embryo transfer results are summarized in Table 1.

Transfers with embryos reconstructed using the 355-F1-derived clonal lines Q2 and Q32 each resulted in one pregnancy to term. A single mummy was recovered from the recipient of Q2-derived embryos. The recipient carrying Q32-derived embryos delivered two live born female piglets, O177-1 and O177-2,

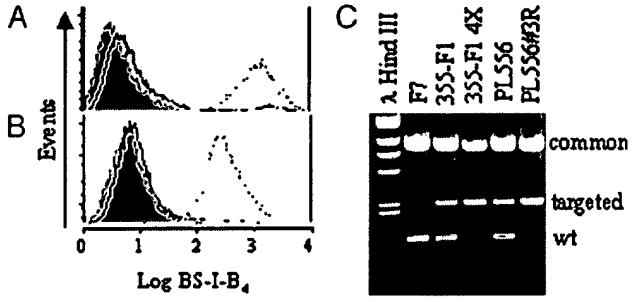


**Fig. 2.** Selection of *GGTA1* null cells from heterozygously targeted fibroblasts. Fibroblasts from heterozygous fetus 355-F1 (*A*) and neonatal piglet PL556 (*B*) were analyzed by flow cytometry for binding to FITC-conjugated Galα-1,3-Gal-specific lectin BS-I-$B_4$. 355-F1 4X and PL556#3R populations (solid lines) were selected four and three times, respectively, by lysis with affinity-purified baboon NAb and complement. Stained cells before selection (broken lines) and unstained selected populations (solid lines) served as positive and negative controls for BS-I-$B_4$ binding. (*C*) Genomic DNA from the above cell populations (and WT fetus F7) was analyzed by PCR by using a forward primer upstream of the selection cassette of the *GGTA1* targeting vector (F527) and a reverse primer (GR2520) downstream of the vector end, as described (11). *Sac*I digestion yields a 2,300-bp band from the targeted GGTA1 allele, a 1,250-bp band from the WT allele, and a 7,900-bp band common to both alleles. The WT band is not detected after NAb/complement selection.

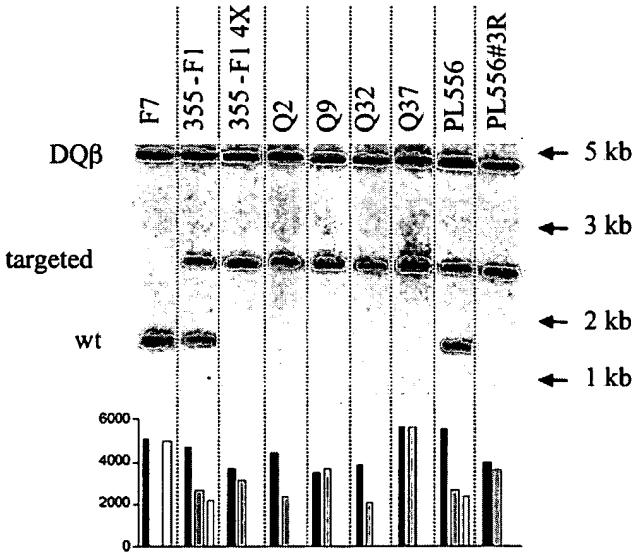


**Fig. 3.** Quantitative Southern blot analysis of NT donor lines. Genomic DNA from the indicated sources was digested with restriction enzyme *Afl*III, Southern blotted, and hybridized simultaneously with a 116-bp probe from exon 9 of the *GGTA1* locus and a 107-bp probe from the porcine SLA *DQβ* locus. The *GGTA1* probe hybridizes a 1.3-kb WT fragment and a 2.3-kb gene-targeted fragment containing an IRES-neo selection cassette. DNA from WT (F7) and heterozygous (355-F1 and PL556) fibroblasts, before NAb/complement selection, served as controls. 355-F1 4X and PL556#3R samples were prepared from cell populations selected four and three times, respectively, with affinity-purified baboon NAb and complement. Q series samples were from clonal cell lines isolated from 355-F1 fetal fibroblasts. Signal quantitation was performed on a Storm 820 PhosphorImager and graphed as absolute values for the *DQβ* locus (■), targeted *GGTA1* allele (▨), and WT *GGTA1* allele (□).

**Table 1. Nuclear transfer with *GGTA1* null cells**

| Cell line | Transfers | Pregnant | Births |
|---|---|---|---|
| Q2 | 4 | 2 | 1 |
| Q9 | 3 | 2 | 0 |
| Q32 | 6 | 3 | 1 O177-1 and -2 born 11/18/02 |
| Q37 | 5 | 3 | 0 |
| PL556#3R | 30 | 7 | 2 PL742–744 born 1/13/03 |

by means of caesarian section. O177-1 weighed 575 g at birth, was healthy, and continued normal growth thereafter. Littermate O177-2 was undersized (275 g) and died shortly after delivery.

Transfers with embryos reconstructed using the null selected cell population from neonate PL556 also resulted in two pregnancies to term. Two dead, late-stage fetuses were obtained by caesarian section from one surrogate. The other surrogate farrowed three healthy male piglets (PL742, PL743, and PL744) weighing 550, 320, and 450 g, respectively.

No evidence of cataract formation, seen previously in *GGTA1* knockout mice (5, 6), or other phenotypic differences between the *GGTA1* null pigs and naturally produced WT miniature swine were observed.

**Molecular Analysis of *GGTA1* Null Pigs.** DNA from the five NT piglets described above was analyzed by quantitative genomic Southern blotting and microsatellite analysis. Piglets O177-1 and O177-2 were found to be hemizygous for the targeted *GGTA1* allele, consistent with their derivation from the Q32 donor cell clone (Fig. 4). Also as expected, both piglets were heterozygous for marker Sw2518.

In contrast to the Q32 derived piglets, all three piglets (PL742–744) derived from the nonclonal PL556 null selected cell line were homozygous for the gene-targeted *GGTA1* allele (Fig. 4). A single heterozygous microsatellite marker, Sw1430, was found from among 26 markers tested in piglet PL556. This centromere proximal marker remained heterozygous in all three

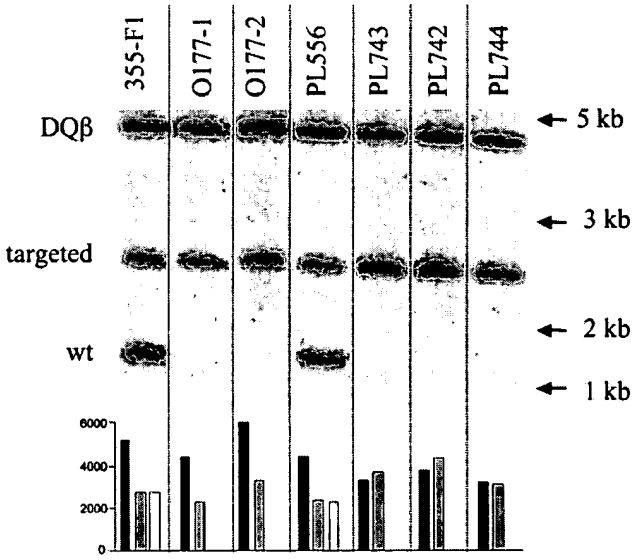


Fig. 4. Quantitative Southern blot analysis of *GGTA1* null piglets. DNA from piglets O177-1 and O177-2 (produced by NT with null fibroblast clone Q32) and piglets PL742–744 (produced by NT with the PL556#3R NAb/complement selected fibroblast population) was analyzed as described in the legend to Fig. 3. DNA from heterozygous 355-F1 and PL556 fibroblasts, without NAb/complement selection, served as controls. Shown are *DQβ* locus (■), targeted *GGTA1* allele (▨), and WT *GGTA1* allele (□).

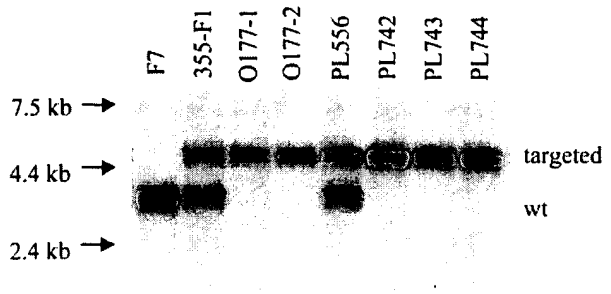


Fig. 5. *GGTA1* expression in fibroblasts from null piglets and progenitors. A Northern blot of poly(A)+ RNA was hybridized to a 1.4-kb probe containing portions of exons 2–9 of the *GGTA1* gene. WT F7 fibroblasts express a 3.6-kb transcript whereas cells from 355-F1 and PL556 heterozygotes express both a 3.6-kb transcript from the WT locus and a 4.7-kb transcript from the targeted locus. Only the 4.7-kb transcript is detected in fibroblasts from the null piglets.

PL556 derived *GGTA1* homozygous piglets. As with homozygous cell clones Q9 and Q37, no nucleotide heterozygosities were found at the junctions of *GGTA1* exon 9 and the G418 selection cassette in the three homozygous piglets (data not shown). Consistent with the hemizygous and homozygous targeted genotypes of these piglets, only RNA compatible with transcription from a targeted locus was observed upon Northern blot analysis (Fig. 5).

**Phenotypic Characterization of Cells from *GGTA1* Null Piglets.** Fibroblast cultures from ear explants of the four surviving null piglets were stained with FITC-labeled BS-I-$B_4$ lectin and examined by flow cytometry for evidence of cell surface Galα-1,3-Gal epitopes (Fig. 6 *A* and *B*). No fluorescence above that obtained

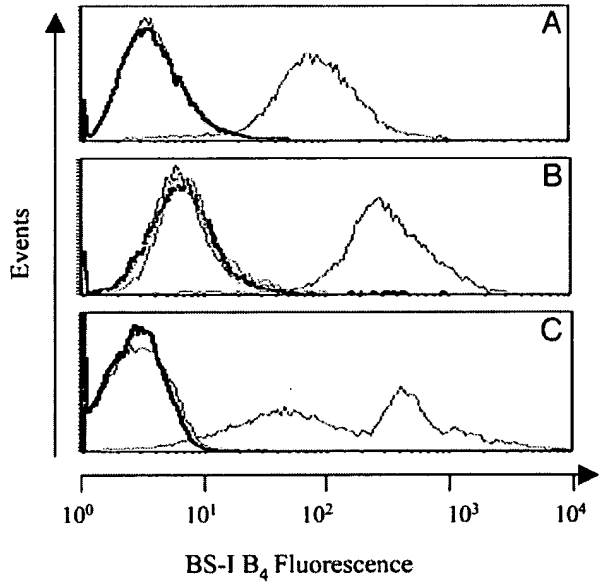


Fig. 6. Flow cytometry analysis of Galα-1,3-Gal epitopes on *GGTA1* null piglets and progenitors using BS-I-$B_4$ lectin. Stained heterozygous or WT cells (red) and unstained cells (black) served as positive and negative controls. (*A*) Stained (blue) and unstained (black) ear fibroblasts from *GGTA1* null piglet O177-1 and fetal fibroblasts from heterozygous progenitor 355-F1 (red). (*B*) Stained ear fibroblasts from null piglets PL742 (orange), PL743 (blue), and PL744 (green); stained (red) and unstained (black) fetal fibroblasts from heterozygous progenitor 355-F1. (*C*) Stained (blue) and unstained (black) multilineage white blood cells from O177-1 at 6 weeks of age and stained WBC from an age-matched WT control (red).

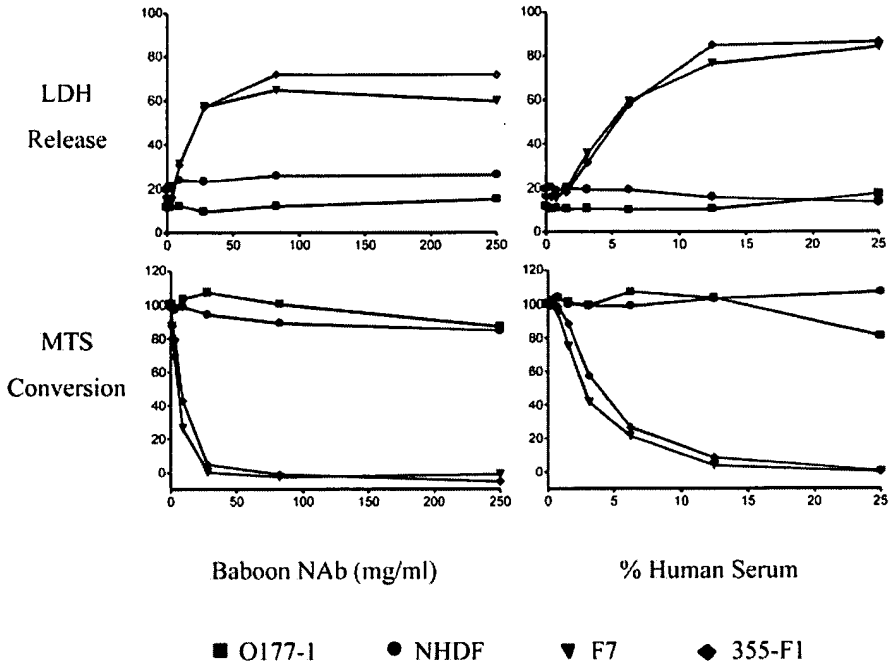


Fig. 7. Complement-mediated lysis of cells after incubation with purified baboon NAb or human sera. Fibroblasts from *GGTA1* null piglet O177-1, WT progenitor fetus F7, and heterozygous progenitor fetus 355-F1 were incubated with the indicated concentrations of affinity-purified polyclonal baboon NAb or heat-inactivated pooled human sera before lysis with rabbit complement. Normal human dermal fibroblasts (NHDF) served as a Gal$\alpha$-1,3-Gal negative control. Release of LDH is expressed as percent of total activity after detergent lysis. Residual metabolic activity, measure as MTS conversion, is expressed as percent of conversion without incubation in complement. Data are the average of three trials.

with unstained fibroblasts was observed. Similar results were obtained with Gal$\alpha$-1,3-Gal-specific mAb M86 (not shown). Epitope expression was also examined on multilineage white blood cells from O177-1 at 6 weeks of age, with none detectable (Fig. 6*C*).

Susceptibility of fibroblasts from null piglet O177-1 to complement-mediated lysis by purified baboon anti-Gal$\alpha$-1,3-Gal antibodies and heat inactivated human sera was assessed by using enzyme release and metabolic activity assays (Fig. 7). With purified antibodies, the $EC_{50}$ for WT and *GGTA1* heterozygous fibroblasts was $<20$ $\mu$g/ml in both assays whereas concentrations up to 250 $\mu$g/ml had no effect on O177-1 cells. Similarly, O177-1 fibroblasts also have far greater resistance to human sera and complement although partial lysis and metabolic inhibition were seen at the highest serum concentration.

## Discussion

Using NT with cells selected for loss of GGTA1 expression from gene-targeted heterozygous cell populations, we have produced healthy null piglets with two distinct LOH genotypes. The stringent selection for loss of function available for this locus permitted efficient selection of *GGTA1* null cell clones from heterozygous fetal cell cultures and sufficient enrichment from heterozygous neonatal cell cultures for use directly in NT.

The WT fetal progenitors for the piglets reported here were two inbred miniature swine, with inbreeding coefficients of 0.86 and 0.91 for fetuses F7 and F501, respectively. Mouse cloning experiments have demonstrated a severe decrease in viability of highly inbred embryos generated entirely by NT and, from many strains, an absolute failure to obtain viable mice (15, 16). Inbreeding in the miniature swine lines, at least to this point, has not resulted in a dramatic decrease in viability although some decrease in NT efficiency in comparison with commercial lines used in unrelated studies seems likely. However, the nature and frequency of spontaneous second allele mutations seems to be greatly influenced by the inbred genetic background. The recovery rate of mutant cells we observe resulting from LOH, $\approx 10^{-4}$, is several orders of magnitude greater than that typically

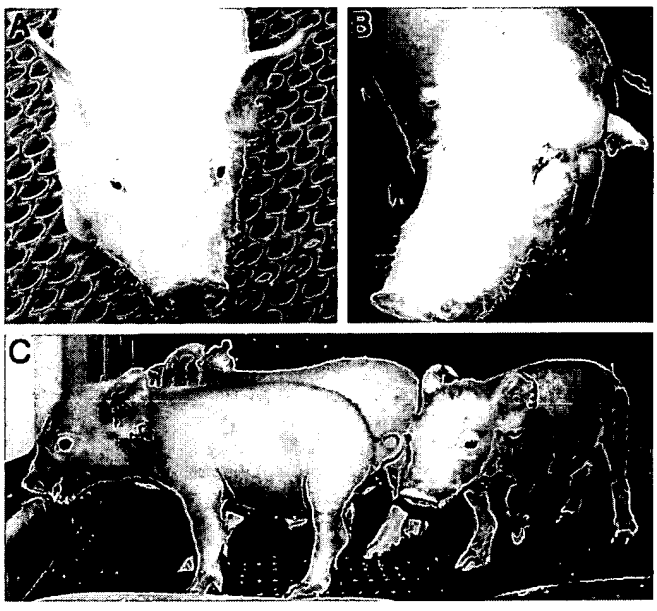


Fig. 8. *GGTA1* null and heterozygous pigs produced by NT. (*A*) Third-round NT piglet O177-1, produced using *GGTA1* null donor cells selected from second-round heterozygous fetus 355-F1 (age 66 days). (*B*) First-round NT pig O212-2 (11), ear fibroblasts from which served as NT donor cells for fetus 355-F1 (age 3 months). Eye and ear defects in this pig are not observed in O177-1, nor were they apparent at 33 days gestation in fetus 355-F1 or any of its 11 clonal littermates. (*C*) Second-round NT piglets PL742–744, produced using *GGTA1* null donor cells selected from first round heterozygous neonate PL556 (age 9 days).

expected in mammalian somatic cells. We have obtained similar results after null selection of fibroblasts from inbred *GGTA1* heterozygous piglets produced by mating (D.K-S. and D.J.J.R., unpublished observations), indicating that the high LOH rate is unrelated to the NT process itself. Furthermore, our results contrast markedly with those obtained in attempts to isolate *GGTA1* null cells on a commercial genetic background, in which mutants were obtained at rates of $<10^{-6}$. Phelps *et al.* (17) used Toxin A from *Clostridium difficile* to select against Gal$\alpha$-1,3-Gal epitope-bearing cells after transfection of heterozygously targeted cells with a second targeting vector. Although no doubly targeted cell clones were obtained, a single clone with a missense mutation in the nontargeted allele was isolated. Sharma *et al.* (18), using a similar antibody and complement selection with heterozygously targeted cells, isolated 11 resistant cell clones from a starting population totaling $2 \times 10^7$ cells. Southern blot analysis of two of these cell clones indicated loss of the nontargeted allele although the mechanism of loss was not further investigated and no pigs were produced by using the antibody resistant lines.

Although not observed in noninbred pigs, the rate of LOH in our inbred pig lines is almost identical to that reported for 2,6-diaminopurine (DAP) selection of spontaneously generated null fibroblasts from heterozygous mice bearing a gene-targeted *Aprt* allele. Shao *et al.* (19), using 129/Sv × C3H/HeJ hybrids, observed LOH in 92 of 113 DAP-resistant clones. In all cases, LOH resulted from somatic crossovers that occurred at various points, with a distribution biased toward the region just proximal to the *Aprt* locus. The propensity for somatic crossing over in this system was subsequently found to be dependent on chromosomal homology because hybrid mice bearing the relevant homologs from distantly related strains yielded much lower frequencies of spontaneous *Aprt* mutant fibroblasts and none of the mutant cell clones recovered were recombination derived (20). Thus, it seems likely that the *GGTA1* homozygous cells used in NT to produce the PL742–44 null piglets also arose through somatic crossing over. However, due to the lack of heterozygous markers in the F501 fetal progenitor, a gene conversion mechanism cannot be formally excluded. It is interesting to note that, if the homozygous donor cells did arise through a somatic crossover, then the pigs would carry a partial uniparental disomy for chromosome 1 distal to the crossover.

In slight contrast to the above *Aprt* studies, Ponomareva *et al.*, using the same gene-targeted *Aprt* allele on a C57BL/6 × DBA/2 background, selected spontaneously generated mutant cell clones with similarly high rates of both somatic crossover events and interstitial deletions (21). We have also recovered both hemizygous and homozygous null lines in all clonal null selection experiments performed with *GGTA1* heterozygous lines. Whereas neither deletional breakpoint seems to map within the *GGTA1* locus in hemizygous piglet O177-1, interstitial deletion mutant cell clones with one or both breakpoints within the *GGTA1* locus have been isolated in selections of other heterozygous fetal and neonatal ear fibroblast lines (D.K-S. and D.J.J.R., unpublished observations). Thus, for deletional events at least, LOH in our inbred derived fibroblast lines is a heterogeneous process.

Heterozygous piglets O212-2 and PL556, from which ear fibroblasts were isolated for second allele mutation selection, were produced early in the program and both were developmentally deficient. PL556 was undersized and died shortly after birth from acute respiratory distress. Although in good health and reproductively sound to date, O212-2 was born with one eye, small earflaps, and no patent ear canals (11). A variety of congenital abnormalities have been reported in cloned pigs (11, 22) as well as other species (22, 23), some of which arise through epigenetic errors in reprogramming (24). Unlike the joint and cardiopulmonary defects not uncommonly observed, the deficits in O212-2 must have arisen at a relatively early stage of development. Whereas it might be assumed that these aberrant phenotypes would be magnified only by additional *in vitro* manipulation and NT, the generation of normal, healthy piglets reported here (Fig. 8) clearly demonstrates that production of normal NT-derived progenitor animals is neither required, nor necessarily advantageous, when performing sequential genetic modifications.

Serial NT has allowed us to produce $\alpha$-1,3-galactosyltransferase null piglets in a considerably shorter timeframe than would be required for standard breeding from heterozygotes. Although the inbred miniature swine lines used here were chosen specifically for their advantages for xenotransplantation (25), they also serve to demonstrate the dramatic effect that genetic background can have on rates of spontaneous somatic mutational events in a large animal model. That somatic recombinational and deletional events are not necessarily associated with other deleterious events suggests that appropriate strain selection or construction may be useful in introducing some genetic modifications by means of NT.

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